In vitro Antioxidant, Cytotoxic and Antidiabetic Activity of Protein Hydrolysates Prepared from Grass Turtle (Chinemys reevesii)

Running title: Evaluation of Bioactivity of Grass Turtle

Md. Serajul Islam¹,², Wang Hongxin¹,²*, Habtamu Admassu³, Amer Ali Mahdi¹,², Ma Chaoyang² and Fu An Wei⁴

¹State Key Laboratory of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi-214122, Jiangsu province, China
²National Engineering Research Center for Functional Food, Jiangnan University, 1800 Lihu Avenue, Wuxi-214122, Jiangsu province, China
³Biotechnology and Bioprocessing Center of Excellence/Department of Food Process Engineering, College of Biological and Chemical Engineering, Addis Ababa Science and Technology University, Addis Ababa, Ethiopia
⁴Guangxi Zhongtaikang Technology Industry Co., Ltd., Nanning-530029, Guangxi, P. R. China

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SUMMARY

Research background. Cardiovascular diseases and diabetes are the biggest causes of death globally. Bioactive peptides derived from many food proteins using enzymatic proteolysis and food processing perform a positive impact on the prevention of these diseases. The bioactivity of grass turtle muscle proteins and their enzymatic hydrolysates has not received much attention, thus this study aims to investigate the antioxidant, antidiabetic, and cytotoxic activities of the enzymatic hydrolysates of grass turtle muscle protein.

Experimental approach. Grass turtle muscle(s) was hydrolysed using four proteolytic enzymes (Alcalase (AH), Flavourzyme (FH), Trypsin (TH), and Bromelain (BH)). The degree of hydrolysis was measured. High performance liquid chromatography (HPLC) was conducted to explore their amino
acid profiles and molecular mass distribution of the hydrolysates. The antioxidant activities were evaluated using various in vitro tests, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Hydroxyl radical (•OH) radical scavenging activities, reducing capacity, chelating (Fe²⁺), and lipid peroxide inhibition activity. Antidiabetic activity was evaluated against α-amylase inhibition and α-Glucosidase inhibition assay. Besides, cytotoxic effect of hydrolysates on human colon cancer (HT-29) cells was assessed.

Results and conclusions. The amino acid composition of the hydrolysates revealed that higher contents of glutamic, aspartic, lysine, hydroxyproline, and hydrophobic amino acids. AH showed significantly the highest inhibition of lipid peroxidation. FH showed the highest lipid peroxidation, strongest radical scavenging activity of DPPH (68.32 %), ABTS (74.12 %), FRAP (0.30 unit), α-glucosidase (61.80 %) and cytotoxic effect on cancer cells (82.26 %) at 550 µg/mL for HT-29 cell line. TH and BH were showed significant (p<0.05) hydroxyl radical activity (92.70 %) and Fe²⁺ metal chelating (63.29 %), respectively. The highest α-amylase (76.89 %) inhibition was recorded by BH and FH.

Novelty and scientific contribution. Enzymatic grass turtle muscle(s) proteins hydrolysates achieved great antioxidant, cytotoxic, and antidiabetic activity. The findings of this study indicated that the bioactive hydrolysates /peptides from grass turtle muscle(s) protein could be potential candidate as ingredients in pharmaceuticals and functional food formulations.

Key words: grass turtle, molecular mass profiles, antioxidant activity, antidiabetic inhibitory capacity, lipid peroxide inhibition, cytotoxic effect

INTRODUCTION

Cardiovascular diseases, chronic obstructive pulmonary disease (COPD), diabetes rheumatoid arthritis, and cancer are the biggest causes of death globally (1). Recently, food-derived bioactive peptides with therapeutic abilities gained an increasing interest. Peptides with specific amino acid sequences that are potent in decreasing and maintaining the onset of diet-related diseases has given particular attention (2). Food-derived protein hydrolysates or peptides as natural food resource materials plays great role in preventing such diseases through inhibiting α-glucosidase and α-amylase, anti-hypertensive, antioxidants activity, anti-proliferative, and anti-microbial effects (2, 3). Enzymatic hydrolysis of proteins is one of the most effective approaches, which can be used to release such bioactive protein hydrolysates or peptides, without affecting their nutritive value.
Enzymatic protein hydrolysates are the smaller peptides derived from the larger polypeptides due to enzymatic action with 2 to 20 amino acids residues (2, 3).

Lipid peroxidation in food and food products is due to rancidity as a result deplorable taste, aroma, and texture as well as shortens shelf life. Naqash and Nazeer (4) elucidated that severe diseases viz., ‘diabetes mellitus (DM), neurological disorders, cardiovascular diseases (CVD), and Alzheimer’s diseases’ may be occur by consuming of oxidative foods. There are artificial antioxidant compounds [Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT), Propyl gallate (PG), and Tert-butylhydroquinone (TBHQ)] that are used lipid peroxidation in food products with under strict regulation owing to their health hazards (5). For this reason, nowadays there is a growing interest to separate and identify anti-oxidative agents from natural resources that can prevent the harmful effects of reactive oxygen species (ROS) (6) including dietary proteins due to their potential health benefits as compared to artificial antioxidants. The bioactivity of peptides is clearly linked with smaller molecular mass (MM), easy absorption, high active, and no negative side effects (7). Lipid peroxidation inhibition and formation of free radicals in foods are important for the prevention of foods from undergoing deterioration (8).

The prevalence of DM is a metabolic disorders which is alarmingly increasing in the world. Ramadhan, Nawas (9) reported type 2 diabetes is increasing at a rate of about 90 to 95 % of cases and predicted to reach 366 million by 2030. Therefore, it is very crucial to minimize the outbreak of DM. The most beneficial therapy for type 2 diabetes is achieved under the optimal blood glucose level after meal. Consequently, α-glucosidase and α-amylase inhibitors are important agents, because α-amylase can break down long chain carbohydrates, whereas, α-glucosidase is catalyzing that can cleavage glucose from disaccharides. As a result, inhibiting these enzymes is effective in delaying glucose absorption. Obesity, free fatty acid peroxidation, and a variety of oxygen free radicals are related to diabetes. Antioxidants can scavenge the body’s peroxides and improve the body’s antioxidant and immunity capability, which helps to the prevention and treatment of diabetes mellitus (10). Many research studies have been explained anti-diabetic peptides from animal sources are underlying in type 2 diabetes prevention (9). On the other hand, cancer is another the most serious single causes for death in both women and men in the world (5). Yaghoubzadeh, Ghadikolaii (11) reported antioxidants are potentially used to prevent and treat diseases including cancer that associated with oxygen species. Additionally, some bioactive peptides can directly kill cancer cells or induce cell apoptosis (12). Although further research is required for the development of effective and less toxic drugs, there is a growing interest on the isolation and characterization of natural anti-tumor agents in food sources in the pharmaceutical industries.
In general, current studies are focused on the practical utilization of numerous aquatic species (13). Grass turtle (*Chinemys reevesii*) is a commercially valuable and protein rich edible aquatic species native in Hong Kong, China, Taiwan, Japan, and Korea. It has been utilized as an ingredient for the traditional Chinese medicines (TCM) (13). Nowadays, it’s highly demanded and commercially cultivated in the above aforementioned countries. The global production of soft-shelled turtles are estimated to 355,000 tons in 2014. It has been reported that *Pelodiscus sinensis* is an aquatic and delicious species because it contains high nutritional value especially high protein and low fat with excellent medicinal values including antioxidant, antidiabetic, anti-cancer as well as blood pressure decreasing compounds (14, 15). Moreover, “genomes of the green sea turtle (*Chelonia mydas*), Chinese soft-shelled turtle (*Pelodiscus sinensis*), and the Western painted turtle (*Chrysemys picta bellii*)” have been testified on their biological and nutritional functions (16). Therefore, in this study, grass turtle proteins were extracted, characterized and protein hydrolysates were evaluated for their biological contribution in regulate the food products quality and health benefits. As far as the authors knowledge is concerned, very limited or scant literature has been previously reported on the specificity of protein properties and production of grass turtle muscle(s) protein hydrolysates (GTPHs) using four proteases (Alcalase, Flavourzyme, Trypsin, and Bromelain) and its, antioxidant, antidiabetic activity and cytotoxic effect on human colon cancer cells of GTPHs. Thus, this study was aimed to optimize the production of GTPHs through the protease enzyme hydrolysis, evaluate nutritional value, antioxidant, antidiabetic activities, and the cytotoxic effect on human colon cancer (HT-29) cell line depending on different treatments.

**MATERIALS AND METHODS**

*Experimental sample*

Grass turtle (*Chinemys reevesii*) is a kind of usual aquatic food in China. For this experiment, grass turtle was obtained from the breeding company of Guangxi zhongtaikang Technology Industry Co., Ltd., Nanning-530029, Guangxi, P.R. China. After consultation with relevant Chinese authorities, it is not considered as an experimental animal and not necessary to issue animal ethics certificate. The grass turtles were slaughtered immediately after it arrived to laboratory and then washed with cleaned water. Sample was put in the fresh ice bag before transferred to Nutrition and Function Factors Food Research Center (Jiangnan University, Wuxi, China). The muscle(s) was separated manually. Finally, selected part was minced, homogenized, and packed into vacuum plastic bags and stored at -20 °C for further experiment.
Chemicals

Alcalase 2.4L (2.4 AU-A/g) from *Bacillus licheniformis* was procured from Nanjing Chengna Chemical Company Limited (Nanjing, China); Bromelain (300 U/mg) from Pineapple; Flavourzyme (20 U/mg) from *Aspergillus oryzae*; Trypsin (250 USP u/mg) from bovine pancreas; α-Amylase (50 u/mg) from *Bacillus subtilis*; α-Glucosidase (50 U/mg) from *Saccharomyces cerevisiae*; p-Nitrophenyl-α-D-glucopyranoside (pNPG, ≥99 %); 2,6-di-tert butyl-4-methylphenol (BHT, ≥99 %); and Ascarbose (≥98 %) were procured from Yuanye Biotechnology Company Limited (Shanghai, China). HT-29 cell was purchased from Chinese Academy of Science Cell Bank (Shanghai, China). All other reagents used were of high purity and analytical grade.

Preparation of protein hydrolysates

Protein hydrolysates (PH) were prepared as described by Noman, Xu (17) with some modifications. Grass turtle muscle(s) was hydrolyzed using four kinds of selected proteases under their optimal conditions as mentioned in Table 1. The pH was set by using 0.025 M of sodium phosphate buffer (pH 6 to 7), and Tris-HCl buffer (pH 7.5 to 9). The enzyme activity was discontinued by heating the mixture at 90 °C for 20 min using a thermostatic water bath (HH-4, Shanghai, China); then, the mixture was immediately transferred to ice bath to cool, centrifuged (ST 40R, Thermo Electron LED GmbH, Germany) at 8,000 × g for 20 min at 4 °C. Finally, the supernatant collected and lyophilized under vacuum at −48 °C (SCIENTZ-10N, Ningbo SCIENTZ Biotechnol Company Limited, Zhejiang, China), and GTPHs were stored at −20 °C for further experiment.

Analysis of degree of hydrolysis (DH)

DH was determined by using formal titration approach as described by Noman, Xu (17) with slight modification. Concisely, 1.5 g of protein hydrolysates was taken and volume made up to 50 g by using ultrapure water. Afterward, the mixture was adjusted to pH 7.0 by using sodium hydroxide solution (0.1 N), and then 10 mL of formaldehyde solution 38 % (V/V) was added and kept for 5 min at 25 °C. Titration was conducted to the end point at pH 8.5 using standard sodium hydroxide (0.1N) solution and the volume consumed was used to calculate the amount of free amino groups (FAG). Total nitrogen (TN) in the sample was examined using the Kjeldahl method by following standard procedures (18). Finally, DH was calculated as follows:

\[
\text{FAG/\%} = \left( \frac{V \times C \times 14.007}{1000 \times 5} \right) \times 100 /1/
\]
DH/\% = \left[ \frac{\% \text{ FAG}}{\% \text{ TN}} \right] \times 100 \quad /2/

where, V= mL of sodium hydroxide (0.1N) used; c= the concentration of the sodium hydroxide solution (0.1 N) used for titration; S= amount of sample (g); and TN= total nitrogen in the sample.

**Average yield and proximate composition**

Average yields of GTPHs were measured according to protocol described by Noman, Qixing (19) and calculated as per the following formula:

\[
\text{Yield/\%} = \frac{\text{GTPH vacuum dried powder (g)}}{\text{Raw sample (g)}} \times 100 \quad /3/
\]

Proximate compositions (moisture, protein, fat, and ash) of GTPHs were investigated using AOAC standard guideline (18). Briefly, moisture content was analyzed by oven air drying method at 105 °C until a constant weight was obtained. Total nitrogen content was estimated by using a standard micro Kjeldahl method. Then, crude protein was calculated by multiplying total nitrogen with a factor of 6.25 (N % × 6.25). Ash content was analyzed by incineration of the samples at 600 °C until a white ash formed in a muffle furnace. The lipid was determined by using macro Soxhlet apparatus (SZG-101, Shanghai, China) with petroleum ether.

**Amino acid composition analysis**

Tryptophan was analyzed by alkaline hydrolysis according to Umayaparvathi, Meenakshi (20) with minor modifications. Briefly, for tryptophan analysis, 100 mg GTPH with 8 mL of 5 mol/L NaOH at 120 °C for 22 h under nitrogen gas and neutralized by 6.67 mL of 6 M HCl. On the other hand, other amino acids were performed using of Noman, Xu (17), the same amount of sample was taken and hydrolyzed with 8 ml of 6 mol/L HCl under nitrogen gas and incubated in an oven at the same temperature and time, neutralized by 4.8 ml of 10M NaOH. Finally, 1 μL of solutions were injected into HPLC analytical column of 250 × 4.6 mm I.D, 5 μm particle size (Agilent Technologies, Palo Alto, California, USA).

**Analysis of molecular mass (MM) distribution**

MM distribution were investigated by gel permeation chromatography using HPLC system (Waters-1525, USA), by using the TSK-GEL 2000 SWXL (300 x 7.8 mm) column (Tosoh, Tokyo, Japan), as described by Noman, Xu (17).
Antioxidants activities

DPPH radical Scavenging activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was evaluated by the protocol of Umayaparvathi, Meenakshi (20). Concisely, 2 mL of protein hydrolysate sample (2-14 mg/mL) was added to 2 mL of 0.16 mM DPPH methanolic solution. The mixture was vortexed for 1 min and left to stand at room temperature for 30 min in dark place, and the absorbance was read at 517 nm. The ability to scavenge the DPPH radical was calculated using the following equation:

\[
\text{DPPH activity/\%} = \frac{A_1 - A_2}{A_1} \times 100
\]

where, \(A_1\) the absorbance of the control (DPPH solution without sample), \(A_2\) the absorbance of the test sample (DPPH solution plus test sample). BHT was used as a positive control.

ABTS radical scavenging activity

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical scavenging assay was analyzed by Chi, Hu (12) method with slight modifications. Briefly, ABTS free radical was generated by mixing the concentration of ABTS stock solution (0.007 M potassium Persulphate and 0.00245 M ABTS). The mixture was kept in the dark at room temperature for 16 h. The ABTS radical stock solution was diluted in 0.005 M phosphate buffered saline (PBS) of pH 7.4 to an absorbance of 0.70±0.02 at 734 nm. Four thousand microliter of diluted ABTS•+ solution was mixed with one hundred microliter of different concentrations of protein hydrolysates (0.5-2 mg/mL), and incubated at 25 °C for 10 min in a dark place. The absorbance value of the mixture was measured at 734 nm, and BHT was used as the positive control. The ABTS capability was calculated by using the equation:

\[
\text{ABTS activity/\%} = \frac{A_1 - A_2}{A_1} \times 100
\]

where, \(A_1\) = absorbance of control and \(A_2\) = absorbance with sample.

Reducing power capacity

The ferric reducing antioxidant power (FRAP) was analyzed according to the procedure by Umayaparvathi, Meenakshi (20) with slight modifications. Concisely, 2 mL of protein hydrolysates was taken at various concentrations (0.5 to 3 mg/mL) and mixed with 2 mL of phosphate buffer (200 mM, pH 6.6) and 2 mL of 1 % potassium ferricyanide was added. The mixture was mixed vigorously by
vortex mixer (XW-80A, Zhejiang, China) for 1 min and incubated at 50 °C for 25 min. Then 1 mL of 10 % TCA was added and mixed, then centrifuged at 10,000 x g for 15 min. After that, the upper layer of solution (supernatant, 2 mL) was collected and mixed with 2 mL of ultrapure water, and 0.4 mL of 0.1 % FeCl₃ was added. Finally, the mixture was incubated for 10 min at 25 °C and absorbance was measured by spectrophotometer (UV-1800PC, Shanghai Mapada Instruments Co., Ltd, Shanghai, China) at 700 nm. BHT was used as a positive control.

Hydroxyl radical scavenging activity

Hydroxyl radical (•OH) scavenging activity of the hydrolysates was analyzed according to modified method of Chi, Hu (12). In this study, our sample concentration was 0.5-2 mg/mL. The mixtures were kept in water bath for 90 min at 25 °C and the absorbance was analyzed at 536 nm by a UV-1800PC Spectrophotometer (Shanghai Mapada Instruments Co., Ltd., Shanghai, China). The reaction mixture without antioxidant was used as the negative control, and a mixture without H₂O₂ was used as the blank. The hydroxyl radical scavenging activity (HRSA) was calculated by the following formula:

\[ HRSA/\% = \frac{A_s-A_n}{A_b-A_n} \times 100 \]

where \( A_s \), \( A_n \), and \( A_b \) are the absorbance the sample, negative control, and the blank after the reaction, respectively. BHT was used as positive control.

Metal chelating

The Fe²⁺ chelating ability of the hydrolysates was evaluated by the method described by Naqash and Nazeer (4) with minor modifications. Concisely, 3200 µL of each sample was prepared at the concentration of 1-20 mg/mL and mixed with 40 µL of 0.002 M FeCl₂, then the mixture was vortexed for 1 min. The mixture was then reacted to 80 µL of 5 mM 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) and incubated at 25 °C for 15 min in a dark place. After incubation, the spectrophotometric absorbance at 562 nm was measured (UV-1800PC, Shanghai Mapada Instruments Co., Ltd, Shanghai, China). BHT was used as a positive control. Chelating activity (%) was calculated by the following equation:

\[ Fe^{2+}/\% = \frac{A_{control}-A_{sample}}{A_{control}} \times 100 \]

where, \( A_{control} \) = absorbance of control and \( A_{sample} \) = absorbance with sample.
The IC$_{50}$ value of the hydrolysates for antioxidant parameter such as DPPH, ABTS, •OH, Fe$_{2}^{2+}$, and BHT were determined by using linear regression analysis (standard calibration curve) from a plot of concentration against % inhibition.

**Lipid peroxide inhibition assay**

The lipid peroxide inhibition activity of the GTPHs was analyzed in a linoleic acid model system according to the method of Chi, Hu (12). Concisely, the protein hydrolysates (25 mg) dissolved in 10 mL of 0.05M PBS (pH 7.0), added in 0.13 mL of linoleic acid, and 10 mL of ethanol (99.7 %). Then the total volume was made up to 25 mL by ultrapure water. The mixture was incubated in a conical flask with a screw cap at 40 °C (±1) in the dark place, and the degree of oxidation was evaluated by measuring ferric thiocyanate values. The reaction solution (100 μL) incubated was mixed with 4.7 mL of 75 % CH$_3$CH$_2$OH, 100 μL of 30 % ammonium thiocyanate (w/v), and 100 μL of 0.02 M ferrous chloride solution in 3.5 % HCl. After 3 min, the thiocyanate value was determined at 500 nm by using a UV-1800PC Spectrophotometer (Shanghai Mapada Instruments Company Limited, Shanghai, China). BHT and α-tocopherol were used as a positive control.

**Antidiabetic activities**

α-amylase inhibition assay

The α-amylase assay was conducted as described by Oseguera-Toledo, de Mejia (21) with minor modifications. The assay mixture containing 500 μL of the protein hydrolysates at different concentration (0.1-2.5 mg/mL) and 500 μL of α-amylase from B. subtilis (1 U/mL) were pre-incubated in test tubes at 37 °C for 10 min in water bath (HH-4, Shanghai, China). Then, 500 μL of 1 % starch prepared in 0.02 mM sodium phosphate buffer at pH 6.9 with 6.7 mM NaCl was added, and the mixture was incubated for another 15 min at 37 °C. The reaction mixture was terminated by adding 500 μL of 3,5-dinitrosalicylic acid (DNS) color reagent to each test tube and placed in boiling water bath at 100 °C for 10 min. The reaction mixture was cooled and diluted with 5 mL of ultrapure water. The absorbance was measured at 540 nm by using a UV-1800PC Spectrophotometer (Shanghai Mapada Instruments Co., Ltd, Shanghai, China). A control, prepared using in sodium phosphate buffer (PH, 6.9) without the test sample and blank was prepared using substrate and buffer without enzyme. Acarbose (1 mg/mL) was used as a positive control. The inhibition (%) was calculated by using following formula:

$$\% \text{ inhibition activity} = 1 - \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Absorbance of control} - \text{Absorbance of blank}} \times 100$$
α-Glucosidase inhibition assay

α-glucosidase inhibition activity was measured as described by Oseguera-Toledo, de Mejia (21) with slight modifications. Reaction mixture containing 50 μL of protein hydrolysates with different concentration (1-5 mg/mL) and 200 μL of α-glucosidase enzyme (1 U/mL in 0.1 M phosphate buffer pH 6.8) was pre-incubated for 15 min at 37 °C in water bath (HH-4, Shanghai, China). After incubation, 50 μL of 5 mM p-nitrophenyl-α-D-glucopyranoside (0.1 M phosphate buffer pH 6.8) was added and further incubated at 37 °C for 40 min. The reaction was terminated by the addition of 1000 μL 0.1M Na₂CO₃ and the α-glucosidase activity was determined spectrophotometrically at 405 nm on UV-1800PC Spectrophotometer (UV-1800PC, Shanghai Mapada Instruments Co., Ltd, Shanghai, China). Acarbose (1 mg/mL) was used as a positive control. The inhibition was calculated by formula.

\[
\% \text{ inhibition activity} = \frac{A_c - A_s}{A_c} \times 100
\]

where, \(A_c\) = absorbance of the negative control and \(A_s\) = absorbance of the sample.

The IC₅₀ value of the hydrolysates to inhibit α-amylase and α-glucosidase activity was determined from the plot of concentration against % inhibition in a linear regression analysis.

Analysis of cytotoxic activity by CCK-8 assay

The cytotoxic activity of GTPHs was evaluated according to the modified method of Li, Zhang (22) with some modifications. The samples were tested against human colon cancer cells (HT-29) by using CCK-8 assay. The cells were cultured in RPMI 1640 medium with 10 % fetal bovine serum (FBS) and 1 % antibiotics at 37 °C in 5 % of CO₂ atmosphere in 96-well microtiter plate (10×10³ cells/well). Stock cultures were sub-cultured two days after harvesting the cells with 0.25 % trypsin-EDTA. GTPHs were dissolved in PBS (0.1M, pH 7.4) and incubated for 24 h, then, GTPHs were diluted with RPMI 1640 medium at different concentrations (100-550 μg/mL) and added them to each well and incubated at 37 °C with 5 % of CO₂ for 24, 48, and 72 h, respectively. The absorbance was analyzed using a microplate reader (EPOCH, BioTek Instruments, Inc, USA) at 450 nm. 5-Fluorouracil (5-FU) was used as positive controls. The percentage of inhibition of cytotoxic activity was measured by following equation:

\[
\% \text{ inhibition} = \frac{A_1 - A_2}{A_1} \times 100
\]

where, \(A_1\) = absorbance of control and \(A_2\) = absorbance with hydrolysates. The protein hydrolysates concentration which gives 50 % growth inhibition was measured and recorded as IC₅₀.
Statistical analysis

All experiments were conducted in triplicate. The results were statistically analyzed by one-way ANOVA using SPSS version 22.0 software (23). Duncan’s Multiple Range Test, the level of statistically significance was considered at p<0.05.

RESULTS AND DISCUSSION

Degree of hydrolysis

The four protease enzymes produced peptides with different DH in various conditions and showed significant correlation under the enzyme concentration and time (Fig. 1). As it can be observed from the results, DH was increased significantly with increasing enzyme concentration and time until the optimum conditions are reached. But increasing the E/S ratio and time over the optimal ratio of Alcalase and Trypsin (2 %, 7 h), Flavourzyme (4 %, 7 h), and Bromelain (5 %, 5 h) had shown no significant variation in the DH. This might be due to the enzyme aggregation, which could be caused by diffusion inhibition of substrates, as a result the saturation of reaction rate. In addition, the small MM of peptides may be attributed to the increase in the DH.

In terms of minced grass turtle muscle(s), the hydrolysates generated by Flavourzyme achieved the highest DH value (28.70 %) at 7 h incubation period (Fig. 1d), followed by Alcalase (15.37 %) at the same incubation time (Fig. 1b), with significant (p<0.05) value. The highest DH value for Bromelain was 12.75 % (Fig. 1e) and 11.01 % was for Trypsin (Fig. 1b) at 7 h incubation. GTPHs showed the higher DH result than the previous findings of Fu, Liu (24) from bovine muscle(s) and porcine plasma. In addition, our Alcalase DH (15.37 %) value was higher than reported by Karami, Peighambardoust (25) who found Alcalase (DH 13.4 %). Therefore, the optimal conditions of this study were chosen as the suitable enzyme–substrate ratio and reaction time for further experiments.

Average yield of GTPH

Average yield of freeze-dried protein hydrolysates under optimal conditions are presented in Table 2. The yield was strongly associated with the DH, and the highest yield (22.76 %) was achieved at DH 28.70 % (FH), followed by 15.03 % yield at DH 15.37 % (AH). Furthermore, 11.93 % yield was obtained for BH at DH 12.75 % and 10.78 % at DH 11.01 % for TH. This different percentage yield may due to enzyme activity and temperature. Alcalase hydrolyzed protein yield was significantly
higher than that reported by Galla, Pamidighantam (26), who found 12.45 % yield of Labeo rohita by using Alcalase hydrolysis.

**Proximate composition**

The proximate composition of both unhydrolyzed and GTPHs are displayed in Table 2. GTPHs contained higher protein (78.66–84.22 %) than unhydrolyzed (73.07 %), but lower fat and ash content. This might be due to the enzymatic hydrolysis, which efficiently reduced the fat content, because of the dissolution of protein during hydrolysis and the centrifugation to separate insoluble and undigested substance. The GTPHs fat (0.38-0.57 %) was lower than previous findings of Noman, Qixing (19) who found 7.92-11.74 % fat from Chinese Sturgeon using Alcalase 2.4L. Therefore, hydrolysis followed by centrifugation can be an effective alternative to market fat products to reduce coronary diseases.

**Amino acid profiles**

Hydrolysis with Alcalase (AH), Flavourzyme (FH), and Trypsin (TH) didn't change too much percentage of most amino acids content, but hydrolysis by Bromelain (BH) has significantly changed (Table 2). The total amino acids of AH, FH, TH, and BH were 72.66, 76.90, 72.27, and 65.63 g/100 g hydrolysate, respectively. The major amino acids in GTPHs were glutamic, aspartic, and lysine, which ranged from 12.15-12.78, 6.70-7.55, and 5.08-6.19 g/100g hydrolysate, respectively. Glutamic acid and aspartic acid both are important amino acids that contribute to palatability and flavor. In addition, alanine, glycine, serine, and threonine have contribution for sweet taste (27). On the other hand, grass turtle muscle(s) (unhydrolyzed) were rich in valine (3.80 %), isoleucine (3.71 %), and proline (3.23 %) than GTPHs. These results might be because all the proteins were not decomposed into peptides with MM distribution during the enzymatic hydrolysis process with a result of few amino acid composition of GTPHs, which were slightly lower than unhydrolyzed (28). However, the essential amino acids were higher than suggested requirements by FAO/WHO (29) for child and adults except the content of tryptophan and phenylalanine+tyrosine amino acids which was slightly lower (for child) (Table 2).

**Molecular mass distribution**

The MM profiles of AH, FH, TH, and BH obtained from grass turtle muscle(s) under the optimum conditions are displayed in Fig. 2. MM distribution was found to be as follows: >10000 Da, 10000-5000 Da, 5000-3000 Da, 3000-2000 Da, 2000-1000 Da, and <1000 Da (Table S1). As clearly shown in Fig. 2, all protein hydrolysates from minced grass turtle muscle(s) were mainly composed
of low MM fractions (<1000 Da), where the percentage distributions were 95.29, 92.25, 90.75 and 78.91 % for FH, AH, BH, and TH, respectively (Table S1). Fu, Liu (24) found 60 % of MM (<1000 Da) for protein hydrolysates of bovine muscle and porcine plasma using 10 different proteases (Alcalase, Flavourzyme, Bromelain etc.), their finding was lower than the results of this current study. Hou, Li (30) reported the available nutritional value which remained in small MM peptides (<1000 Da), contributing to the rich dietary proteins. Additionally, it has been reported that low molecular mass peptides highly contribute to enhance bioactivity such as antioxidant activity (31,32).

**Antioxidants activities**

**DPPH free radical scavenging activity**

The DPPH free radical scavenging ability of GTPHs (protein hydrolysates) with various enzymatic treatments are displayed in Fig. 3a. The results showed that DPPH radical scavenging activity of the GTPHs in different concentrations indicated that the FH achieved the highest DPPH activity of 68.32 % (14 mg/mL), followed by AH (57.45 %) at the same concentration. On the other hand, TH had significantly (p<0.05) higher DPPH radical inhibitory power than BH. The current results of the GTPHs may have connections with some amino acid composition, DH, and MM of peptides that are electron donors and may react with free radicals to convert them to more stable products. Park, Je (33) reported the amino acids, including threonine, valine, isoleucine, and hydrophobics, strongly contributed for the positive influence of the DPPH scavenging activity.

The IC$_{50}$ values of FH was 9.36±0.21 mg/mL, which was stronger than the other enzymatic hydrolysates such as AH (12.21±0.17 mg/mL), TH (12.21±0.31 mg/mL), and BH (12.38±0.29 mg/mL). However, DPPH power of GTPHs were significantly lower than that of the standard BHT (IC$_{50}$= 0.16±0.00 mg/mL). The percentage of DPPH activity was higher than Housefly larvae hydrolysate using Alcalase 2.4 L and Flavourzyme (34). The current study noticeably showed that FH had the highest DPPH radical scavenging activity because it contained more electrons for donating and converting the free radicals into the more stable products by terminating the radical chain reactions.

**ABTS free radical scavenging activity**

ABTS radical approach is an excellent tool for evaluation of the anti-oxidative activity, which the radical is slaked to form ABTS radical complex (35). As clearly shown in Fig. 3b, the highest inhibitory activity (74.12 %) of ABTS$^+$ was achieved by FH followed by AH (73.49 %) at the concentration of 2 mg/mL, with no significant difference (p ≥0.05). However, BH (65.99 %) and TH (63.40 %) showed significant differences (p<0.05) of scavenging activity than FH and AH at the
concentration of 2 mg/mL. This research results were in agreement with Chi, Hu (12), who found 85.10 % at the concentration of 2.5 mg/mL of blood clam (Tegillarca granosa) muscle. Hassan, Xavier (36) reported that some amino acids (cysteine, tryptophan, tyrosine, and histidine) showed better ABTS scavenging activity. It was also found that the active peptides mainly with small molecular mass were responsible for antioxidant activity (32). These findings were closely related with the current research results (Table 2 and Fig. 2). Their IC$_{50}$ values for ABTS radical of AH, FH, BH, and TH were 0.85±0.03, 0.91±0.01, 1.41±0.03, and 1.43±0.07 mg/mL, respectively, which were lower in antioxidant activity than BHT (IC$_{50}$=0.09±0.004 mg/mL). The result exhibited that the AH and FH both are showing better ABTS radical activities, which could be used as potential natural antioxidants.

Reducing power

The ferric ion reducing antioxidant power of GTPHs are revealed in Fig. 3c. The results clearly showed that reducing power of GTPHs was increased significantly (p<0.05) as the concentration of protein hydrolysates increased. The higher absorbance value was recorded in FH (0.30) followed by BH (0.25) at the concentration of 3 mg/mL showing significant (p<0.05) difference. Beside, AH and TH showed the lower absorbance of 0.180 and 0.175 with no significant (p ≥0.05) difference. Thus, the increase in absorbance indicated the higher reducing power of hydrolysates. The strong capability of FH may be attributed due to the presence of H$^+$ (protons and electrons) generated during peptide cleavages. The reducing power were higher in GTPHs than Spanish mackerel skin hydrolysate (37) and hemoglobin hydrolysate (38). Although, the results of this study were lower than the commercial BHT which is 0.57 at the concentration of 0.1 mg/mL, these GTPHs can be used as a potential reducing agent. In the present study, reducing power ability was different in all enzymatic treatments, possibly due to enzyme specificity to hydrolyze the substrate and properties of the chemicals used for extraction of protein hydrolysate from grass turtle muscle(s). In addition, Cumby, Zhong (39) found the significant difference in reducing capacities of hydrolysates prepared, which might be due to the substrate specificity of enzymes.

Hydroxyl radical scavenging activity

Hydroxyl radical (•OH) is a highly capable of attacking and destroying the biomolecules such as proteins, amino acids, and lipids in living cells (40). Thus, hydroxyl radical scavenging activity evaluation is an important parameter which can provide valuable information on the antioxidant activities of peptides. The hydroxyl radical scavenging activity of GTPHs are presented in Fig. 3d.
Potentially, significant (p<0.05) scavenging activity was observed in TH (92.70 %) followed by FH (82.85 %) at concentration of 2 mg/mL, but AH and BH exhibited scavenging activity without significant difference (p≥0.05) at the same concentration. The IC₅₀ values for their hydroxyl radical scavenging activity were 0.69±0.02 mg/mL for TH, 0.83±0.05 mg/mL for FH, 0.94±0.02 mg/mL for AH, and 1.14±0.07 mg/mL for BH, which were lower in hydroxyl radical scavenging activity as compared with BHT standard (IC₅₀= 0.24±0.01 mg/mL). As it can be seen from the results that BH exhibited higher IC₅₀ value, showing a lower hydroxyl radical scavenging activity than the other enzymatic treatments. In general, in this study, GTPH showed an excellent •OH scavenging activity, which could be used as a scavenger for reducing the damage induced by hydroxyl radicals in biosystems, food and pharmaceutical products.

Metal (Fe⁺²) chelating activity

Metal chelating act as catalysts that reduce the accessibility of transition metals and protect the radical mediated oxidative chain reactions in food or biological properties. Therefore, they are necessary to improve the food quality, food safety and stability as well as human health benefits (41). The chelating activity of GTPHs with different enzymatic hydrolysis is displayed in Fig. 3e. As shown in the Fig., the Fe⁺² chelating activity increased significantly (p<0.05), the highest result achieved was 63.29 % for BH followed by 58.14 % for FH. The lowest value was 52.19 % and 51.46 % for AH and TH, respectively. These results may be occurred due to the attribution of the incapability of small peptides that form the complex with metals. Hamzeh, Benjakul (42) reported that metal chelating activity was decreased with increasing DH. Also Noman, Qixing (19) found that metal ion chelating activity was ≥52 % at 50 mg/mL of Chinese Sturgeon hydrolysate which was lower than the GTPHs. The IC₅₀ value of the GTPHs were obtained in the increasing order as for BH (16.36±0.31 mg/mL), for FH (17.95±0.50 mg/mL), for AH (18.33±0.74 mg/mL), and for TH (18.89±0.27 mg/mL) with significant differences between BH and FH. However, no significant (p≥0.05) difference was observed between AH and TH. Their IC₅₀ values were lower than the positive control BHT (3.87±0.12 mg/mL). However, the GTPHs can play a potential role as a natural antioxidant source.

Lipid peroxidation inhibition

Lipid oxidation is the main cause of food spoilage. Lipid peroxidation inhibition activity is an important indicator for evaluating antioxidant activity of protein hydrolysates or peptides, which initiates a sequence of reactions that can generate ketones, aldehydes and other potentially toxic substances (43). Thus, the lipid peroxidation inhibition activities of AH, FH, TH, and BH were
evaluated at the concentration of 1 mg/mL using a linoleic acid system and the results achieved after seven days of incubation period are indicated in Fig. 4. The highest absorbance value of the negative control (without antioxidant) indicated the highest level of linoleic acid peroxides. Compared with the negative control, the positive control (α-tocopherol and BHT) of AH, FH, TH, and BH strongly inhibited lipid peroxidation in the system during the incubation time.

The incubation period of 1 to 7 days showed natural antioxidant α-tocopherol was significantly (p<0.05) lower inhibition activity than of AH, FH, TH, and BH, but the activity of synthetic BHT was higher than all hydrolysates, except the 3 days incubation of AH, FH, but not for TH and BH. As illustrated in Fig. 4, AH was the highest activity than other hydrolysates at 1, 5, and 7 days, but in 7 days with no significant (p ≥0.05) difference. Similarly, FH was showing higher at 3 and 4 days. In the 6th days incubation activity, the results of the GTPHs was in the following sequence: TH>AH>FH>BH. The BH capability to inhibit linoleic acid oxidation at all incubation days (except 2nd days) may be due to depletion of free electrons (6). In addition, aromatic and/or hydrophobic amino acids (Table 2), which could lead to more interactions between peptides and fatty acids by increasing the peptides solubility in lipids, as a result may increase the oxidation prevention (44). Our results were supported by Chi, Hu (12). However, this study results displayed that had superior inhibition of linoleic acid oxidation when compared to rapeseed protein hydrolysates lipid peroxidation inhibitory activity at the same concentration (6).

**Antidiabetic activities**

In vitro α-amylase inhibitory activity

Alcalase, Flavourzyme, Trypsin, and Bromelain hydrolysates expressed a significant inhibition against α-amylase enzymatic activity (Fig. 5a). The strongest α-amylase inhibitory ability obtained was 76.89 % for BH, followed by FH (58.79 %), AH (54.25 %), and TH (51.03 %) at the concentration of 2.5 mg/mL. This may due to peptides having branched chain amino acids (tryptophan, phenylalanine, lysine, tyrosine, and valine) and cationic residues preferably bound to α-amylase (45). The IC_{50} value of the BH was 1.13±0.02 mg/mL followed by FH (2.12±0.05 mg/mL), AH (2.31±0.04 mg/mL), TH (2.41±0.07 mg/mL). However, these values were lower in compared with the standard acarbose (IC_{50}= 0.71±0.03 mg/mL). This may due to acarbose is a purified artificial inhibitor of α-amylase, whereas the GTPHs were the mixtures of peptides and probably some non-protein components are present together. Connolly, Piggott (46) described that type 2 diabetes management using acarbose drug was associated with negative side effects (such as abdominal dissention,
meteorism, flatulence and probable diarrhea). Thus, GTPHs can play great role as natural source of antidiabetic agent to substitute acarbose if the hydrolysates can be further purified.

**In vitro α-glucosidase inhibitory activity**

The α-glucosidase inhibitors have an important role to the prevention of type 2 diabetes, that can decrease the absorption of carbohydrates and reduce the postprandial hyperglycemia (47). As illustrated in Fig. 5b, the α-glucosidase inhibitory activity significantly (p<0.05) increased with increasing the concentrations of 1-5 mg/mL. The IC<sub>50</sub> values against α-glucosidase inhibitory activity were FH (3.76±0.08 mg/mL), TH (4.46±0.15 mg/mL), AH (4.51±0.03 mg/mL), and (4.91±0.10 mg/mL), in which the inhibitory activities of the hydrolysates were much lower than the acarbose (IC<sub>50</sub>=1.44±0.01 mg/mL). All hydrolysates had good inhibitory activity on α-glucosidase, whereas FH inhibitory activity was stronger than the others (AH>BH>TH). Yu, Yin (48) found that α-amylase and α-glucosidase inhibitors both were compounds that help in control of diabetes by declining the absorption of glucose.

**Cytotoxic effect of GTPHs**

The inhibitory effect observed after 24, 48, and 72 h of incubation at the same concentration level are displayed in Fig. 6. As illustrated in Fig. 6b, the maximum HT-29 cell inhibition was obtained by FH (82.26 %) with the concentration of 550 µg/mL at 72 h incubation. The cytotoxic effect of hydrolysates on HT-29 cells was increased with increasing incubation time. The incubation periods of 48 and 72 h were better, as compared with 24 h incubation period. Hence, the results indicated that the GTPHs showed a perceptible dose and time-dependent cytotoxic effect on colon cancer cells. The present findings were in agreement with the findings of Umayaparvathi, Arumugam (5) in which they studied the hydrolysates of Oyster (*Saccostrea cucullata*) on the cytotoxicity against human colon cancer cell lines (HT-29) in time. In this study, the cytotoxic activity of grass turtle muscle(s) hydrolysates were higher than that of Kim (49), who discovered stomach (AGS), human colon (DLD-1, and cervical (HeLa) cancer cells at 1 mg/mL from Solitary Tunicate hydrolysate and also Alemán, Pérez-Santin (50) observed cytotoxic activity on Alcalase squid gelatin hydrolysate for MCF-7 cell lines (41.64 %) at concentration of 1 mg/mL.

Furthermore, as observed in Fig. 6f, the IC<sub>50</sub> values of the GTPHs were better for active hydrolysates than the commercial standard drug 5-FU after the incubation of 24 h. But AH, FH, and TH after 24 h and 72 h incubation period showed slightly lower value than that of active standard drug 5-FU. Similarly, after 48 h incubation, FH had strong activity (IC<sub>50</sub>= 172.49±4.10 µg/mL), followed by
TH (IC$_{50}$= 236.04±8.06 µg/mL). The findings of the present study was slightly lower than the findings of Umayaparvathi, Arumugam (5), who reported on the cytotoxic activity of human colon cancer cells (HT-29) with IC$_{50}$ values 90.31±0.45 µg/mL (except BH at 72 h) on HT-29 cell. But our experiment value was higher than by Karami, Peighambardoust (25) who obtained Alcalase (IC$_{50}$=12.94 mg/mL) of lung cancer cell line. These significant variations of GTPHs on cytotoxic activity may due to various chemical compositions of proteins originated from differences in enzyme specificity, extraction as well as the use of cancer cells. The results of the present study also confirm the capability of hydrolysates to protect normal cells.

CONCLUSIONS

Different enzymes lead to different MM distribution, DH, and amino acid composition, which greatly influence the cytotoxic, antioxidant, and antidiabetic activities potential of protein hydrolysates. The GTPHs showed antioxidant activities (DPPH, ABTS, and reducing power), which were significantly higher in FH, whereas hydroxyl radical in TH and iron metal chelating in BH. AH and FH strongly inhibited linoleic acid oxidation than other hydrolysates. Bromelain hydrolysates demonstrated the strongest α-amylase and Flavourzyme hydrolysates showed α-glucosidase inhibitory capabilities. Moreover, FH had the highest cytotoxic effect against colon cancer cell line (HT-29). From these results, it can be suggest proposed that grass turtle muscle(s) hydrolysates can be used as alternatives of new natural materials in the development of functional foods with potential cytotoxic, antidiabetic, and antioxidant activities.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.
ETHICAL STATEMENT

This research article does not contain any studies with animals or human participants performed by any of the authors.

AUTHORS’ CONTRIBUTION

M.S. Islam contributed to the conception of the research design, performed the experiment and wrote the manuscript. W. Hongxin Supervision entire work and performed to finalizing the manuscript. H. Admassu contributed to editing the manuscript. A. Mahdi performed the data analyses. C. Ma helped perform the analysis with constructive discussions. F. Wei contributed to the formal data analysis and raw materials collected. All authors have read and approved the final manuscript.

ORCID ID

M.S. Islam https://orcid.org/0000-0002-0495-2733
H. Wang https://orcid.org/0000-0002-7616-9537
H. Admassu https://orcid.org/0000-0001-9933-3988
A. Mahdi https://orcid.org/0000-0003-4750-1753
F.A. Wei https://orcid.org/0000-0002-6026-0176

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Fig. 1. Effects of different conditions on the degree of hydrolysis: a) 1% E/S (Enzyme/Substrate), b) 2% E/S (Enzyme/Substrate), c) 3% E/S (Enzyme/Substrate), d) 4% E/S (Enzyme/Substrate), and e) 5% E/S (Enzyme/Substrate). Different small letters (a–d) within each assay indicate significant differences (p < 0.05). Data are expressed as mean ± S.D. of triplicate. S.D. = standard deviation.

Fig. 2. Molecular weight distribution of grass turtle muscle protein hydrolysates (GTPHs), The spectra was obtained by high performance size exclusion chromatography (TSKgel 2000 SWXL column), hydrolyzed by using Alcalase (AH), Flavourzyme (FH), Trypsin (TH), and Bromelain (BH). tR = Retention time.

Fig. 3. The antioxidant activities of grass turtle muscle protein hydrolysates (GTPHs) at the different concentrations including a) DPPH radical scavenging activity, b) ABTS radical scavenging activity, c) Reducing power at 700 nm, d) Hydroxyl radical scavenging activity, and e) Fe²⁺ chelating activity. All results are presented as mean ± SD (n = 3) of triplicate measurements, with different alphabets (a–d) have mean values that are significantly different at p < 0.05. γ/(mg/mL) = concentration (mg/mL).

Fig. 4. Lipid peroxidation inhibition activity of grass turtle muscle protein hydrolysates (GTPHs) at the various concentrations. All results are expressed as mean value ± S.D. of triplicate measurements.

Fig. 5. In vitro antidiabetic activities of grass turtle muscle protein hydrolysates (GTPHs) using a) α-amylase and b) α-glucosidase compared with Alcalase (AH), Flavourzyme (FH), Trypsin (TH), and Bromelain (BH). Results are presented as mean ± SD (n = 3). Bars at the same concentration but with different small letters (a–d) are significantly different at p < 0.05.

Fig. 6. Cytotoxicity effects of grass turtle muscle protein hydrolysates (GTPHs) on HT-29 against different concentrations of 100-550 µg/mL cell lines over a period of 24, 48, and 72 h: a) Alcalase (AH), b) Flavourzyme (FH), c) Trypsin (TH), d) Bromelain (BH), e) 5-Fluorouracil (5-FU), and f) IC₅₀ values. The values are given as mean ± SD (n = 3). Bars with letters (a–e) differ significantly with each other (p < 0.05).
Table 1. The hydrolysis conditions for preparation of protein hydrolysates from grass turtle

<table>
<thead>
<tr>
<th>Hydrolysis conditions</th>
<th>Proteases</th>
<th>Alcalase</th>
<th>Flavourzyme</th>
<th>Trypsin</th>
<th>Bromelain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation temperature/°C</td>
<td>55</td>
<td>50</td>
<td>60</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>8.0</td>
<td>7.5</td>
<td>8.0</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>(^a)(m(enzyme)/m(substrate))/%</td>
<td>1, 2, 3, 4, 5</td>
<td>1, 2, 3, 4, 5</td>
<td>1, 2, 3, 4, 5</td>
<td>1, 2, 3, 4, 5</td>
<td></td>
</tr>
<tr>
<td>(m)(solid)/V(liquid))/(g/mL)</td>
<td>1:1</td>
<td>1:1</td>
<td>1:1</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>(t)(incubation)/h</td>
<td>1,3,5,7</td>
<td>1,3,5,7</td>
<td>1,3,5,7</td>
<td>1,3,5,7</td>
<td></td>
</tr>
<tr>
<td>Inactivation temperature/°C</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Inactivation time/ min</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
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</tr>
</tbody>
</table>

\(^a\) Enzyme to substrate ratio.

Table 2. Proximate composition (%), yield (%), and amino acid composition (g/100 g sample) of unhydrolyzed and protein hydrolysates

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Unhydrolyzed</th>
<th>AH</th>
<th>FH</th>
<th>TH</th>
<th>BH</th>
<th>(^e) FAO requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>(73.07±1.32)(^a)</td>
<td>(80.89±0.42)(^b)</td>
<td>(84.22±1.12)(^c)</td>
<td>(79.72±1.76)(^d)</td>
<td>(78.66±1.28)(^b)</td>
<td></td>
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<tr>
<td>Moisture</td>
<td>(8.43±0.25)(^a)</td>
<td>(6.56±0.13)(^b)</td>
<td>(6.95±0.22)(^c)</td>
<td>(6.68±0.27)(^c)</td>
<td>(7.22±0.34)(^b)</td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>(3.20±0.07)(^a)</td>
<td>(0.57±0.02)(^b)</td>
<td>(0.38±0.01)(^c)</td>
<td>(0.55±0.02)(^c)</td>
<td>(0.38±0.01)(^c)</td>
<td></td>
</tr>
<tr>
<td>Ash</td>
<td>(8.08±0.27)(^a)</td>
<td>(5.69±0.27)(^b)</td>
<td>(4.52±0.35)(^d)</td>
<td>(6.92±0.17)(^b)</td>
<td>(6.64±0.29)(^b)</td>
<td></td>
</tr>
<tr>
<td>Yield</td>
<td>-</td>
<td>(15.03±0.42)(^b)</td>
<td>(22.76±0.64)(^d)</td>
<td>(10.78±0.11)(^d)</td>
<td>(11.93±0.33)(^b)</td>
<td></td>
</tr>
</tbody>
</table>

Non-essential amino acid

<table>
<thead>
<tr>
<th>Essential amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
</tr>
<tr>
<td>Histidine</td>
</tr>
<tr>
<td>Methionine+cysteine</td>
</tr>
<tr>
<td>Phenylalanine+Tyrosine</td>
</tr>
<tr>
<td>Threonine</td>
</tr>
<tr>
<td>Isoleucine</td>
</tr>
<tr>
<td>Leucine</td>
</tr>
<tr>
<td>Lysine</td>
</tr>
<tr>
<td>Valine</td>
</tr>
</tbody>
</table>

Non-essential amino acid

\(^e\) FAO requirements

Child: 1.3, 1.6, 1.9

Adult: 1.3, 1.6, 1.9

FAO = Food and Agriculture Organization
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>AH</th>
<th>FH</th>
<th>TH</th>
<th>BH</th>
<th>FAO/WHO (29)</th>
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<tbody>
<tr>
<td>Taurine</td>
<td>0.26±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.36±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.45±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.50±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.52±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>6.23±0.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.84±0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.55±0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.41±0.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.70±0.23&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>11.38±0.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.78±0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.57±0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.31±0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.15±0.47&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Serine</td>
<td>2.69±0.04b&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.70±0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.81±0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.49±0.08&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.58±0.09&lt;sup&gt;cd&lt;/sup&gt;</td>
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<tr>
<td>Glycine</td>
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<td>4.88±0.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.77±0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.98±0.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.92±0.15&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Arginine</td>
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<td>4.94±0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.43±0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.53±0.13&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Alanine</td>
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<td>5.57±0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.47±0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.35±0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.78±0.10&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>Proline</td>
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<td>3.19±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.79±0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.53±0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.21±0.13&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>1.04±0.05&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.07±0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.20±0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.35±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.77±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Hydrophobic</td>
<td>27.06</td>
<td>26.98</td>
<td>28.91</td>
<td>25.74</td>
<td>23.69</td>
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<tr>
<td>Aromatic</td>
<td>5.04</td>
<td>4.54</td>
<td>4.74</td>
<td>4.09</td>
<td>4.14</td>
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<tr>
<td>(+) Charged</td>
<td>11.37</td>
<td>11.84</td>
<td>12.94</td>
<td>12.16</td>
<td>10.2</td>
</tr>
<tr>
<td>(−) Charged</td>
<td>17.61</td>
<td>19.62</td>
<td>20.12</td>
<td>19.72</td>
<td>18.85</td>
</tr>
</tbody>
</table>

<sup>a</sup> FAO/WHO (29). Data are expressed as (Mean ± S.D., n = 3). Values in the same row followed by different Latin letters (<sup>a</sup>−<sup>d</sup>) show significantly difference at p<0.05.

Abbreviation: AH=Alcalase hydrolysed, FH=Flavourzyme hydrolysed, TH=Trypsin hydrolysed, BH=Bromelain hydrolysed, FAO=Food and Agriculture Organization.
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Fig. 1

**Fig. 1**

**a)**

**b)**
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Fig. 3
Fig. 4
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Fig. 5
Please note that this is an unedited version of the manuscript that has been accepted for publication. This version will undergo copyediting and typesetting before its final form for publication. We are providing this version as a service to our readers. The published version will differ from this one as a result of linguistic and technical corrections and layout editing.

Fig. 6
Supplementary material Table S1

<table>
<thead>
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<th>MW (Da)</th>
<th>Content (%)</th>
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<tr>
<td></td>
<td>AH (a)</td>
<td>FH (b)</td>
<td>TH (c)</td>
<td>BH (d)</td>
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<td>&gt;10000</td>
<td>0.19</td>
<td>0.43</td>
<td>0.42</td>
<td>0.45</td>
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<td>1.60</td>
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