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Original Research Article

Influence of *In vitro* Digestion on Antioxidative Activity of Coconut Meat Protein Hydrolysates

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Abstract

Purpose: To investigate the antioxidative stability of coconut meat protein hydrolysates (CMPHs) in the gastrointestinal tract, and evaluate the changes in antioxidant activity, amino acid composition and molecular weight distribution of CMPHs during gastrointestinal (GI) digestion**Methods:** A two-stage *in vitro* digestion model (pepsin treatment for 2 h followed by pancreatin treatment for 2 h, both at 37 °C) was used to simulate the process of GI digestion to determine changes in antioxidant activities, namely, 1,1- diphenyl-2-picrylhydrazyl and hydroxyl radical scavenging and reducing power, of CMPHs previously prepared by papain digestion.**Results:** Based on the *in vitro* pepsin–pancreatin simulated GI digestion, it was found that there were more free amino acids and smaller oligopeptides with MW < 500 Da in the final GI digests. Compared with blank, enzymatic breakdown of the GI digests increased their hydroxyl (by 11.8 %) and reducing power (by 71.8 %).**Conclusion:** CMPHs are high value-added antioxidants and possess a potential capacity to resist gastrointestinal digestion, which makes them promising ingredients for formulation of functional foods.**Keywords:** Coconut meat protein, Hydrolysates, Simulated gastrointestinal digestion, Antioxidative stability, Oligopeptides, Functional foods

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INTRODUCTION

Considerable evidences had confirmed that free radicals were involved in the oxidation of lipids, and oxidative decomposition of unsaturated fatty acids was the primary cause of the development of undesirable off-flavours, odours and potentially toxic reaction products [1,2]. Moreover, free radicals are believed to play a significant role in the occurrence of many chronic diseases, such as diabetes, cardiovascular diseases, neurodegenerative disorders and cancer [3,4]. Therefore, there is a growing interest in finding antioxidants from natural sources including some

dietary protein compounds which may have less health hazard than synthetic antioxidants.

Protein hydrolysates have been found to possess strong *in vitro* antioxidant activities, such as soy, *Sphyrna lewini* muscle, loach (*Misgurnus anguillicaudatus*) and pea [5-8]. The gastrointestinal (GI) tract is known to be a major oxidation site in the human body [9]. The net effectiveness of foodborne antioxidants depends on their actual level in the GI tract. Thus, there is a need to investigate the stability of antioxidants in the GI tract.

Coconut (*Cocos nucifera* L.) is a monocotyledonous plant of the Arecaceae family widely distributed in the tropical areas of Asia, Africa, Latin America, and the Pacific region. Coconuts are an important commodity in Guangdong, China. Coconut fruit is composed of about 38.5 % shell, 51.7 % kernel and 9.8 % water [10]. Fresh coconut meat ("kernel") is 35.2 % fat, 3.8 % protein and 40 % moisture [11]. Coconut oil and protein can be extracted from dried and fresh coconut meat. Even though the protein content of coconuts meat is low (4 %), the large amount of coconut grown could make the recovery of the protein economically attractive. An estimated 1.6 million tons of protein from fresh coconut or 0.4 million tons from copra on a world-wide basis is potentially available for human consumption. As well as being highly nutritious, coconut meat protein also has excellent functional properties with foaming and emulsifying capacities greater than many legume and oilseed proteins, which is already used extensively as a food ingredient [12].

Moreover, previous studies have shown that coconut meat protein had beneficial anti-oxidative and cardioprotective effect on alcohol and isoproterenol treated rats as well as on human volunteers [13,14]. However, the protein fraction is either discarded or used as animal feed presently. It is of great significance, therefore, to find an effective way to utilise this protein source.

The results from our previous studies have established that coconut meat hydrolysates and peptides are potential antioxidants for food applications [15]. However, the degradation and the performance of coconut meat protein hydrolysates during gastrointestinal digestion are unclear. In this work, the influence of *in vitro* GI digestion on the stability and effectiveness of protein hydrolysates from coconut meat was investigated.

EXPERIMENTAL

Materials and chemicals

Fresh matured coconuts (hybrid Mawa variety) used in these experiments were purchased from a local market in Zhanjiang, China. The matured coconuts consisted of hard outer shell which enclosed the test. After cracking the nuts and discarding the coconut water, the meat was removed from the shells and manually pared, and only the pared meat was used for all experiments. The fat content of coconut meat

(53.10 %), protein content (6.38 %), and average moisture (46.36 %) on wet basis were determined by the standard Association of Official Analytical Chemists method (AOAC, 1984). Coconut meat was separated, pulverized and defatted using petroleum ether (60 - 80 °C) for 2 - 3 h. The defatted meal was grounded to pass through a 15 mm screen sieve. The ground meal was freeze-dried and stored at -20 °C until needed. Alcalase 2.4 L (EC 3.4.21.62, from *Bacillus licheniformis*, 2.4 AU/g) was obtained from Baiao Biochemistry Co. (Jiangmen, China). Pepsin (2500–3500 units/mg protein), pancreatin (8 x the standard USP unit), 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade and obtained from Zixing Reagent Co. (Guangzhou, China).

Preparation of coconut meat protein

Defatted material was stirred with pH 8.0 phosphate buffer in large containers using mechanical stirrer for 24 h at 4 °C. The saline extract was collected and by adjusting the pH 4.0 with dilute HCl solution, the proteins were precipitated. The solution was centrifuged at 5000 r/min for 10 min and the precipitated globulin fraction was collected, washed with distilled water 3 times, freeze-dried and stored at -20 °C until needed.

Preparation of coconut meat hydrolysates (CMPHs)

Ten grams of coconut meat protein were mixed with 100 mL of distilled water and homogenised at a speed of 8000 rpm for 5 min using a basic homogeniser (T25, Ika, Staufen, Germany). The homogenate was hydrolysed with Alcalase at 50 °C for 7 h to obtain a hydrolysate that was previously shown to have strong antioxidative activity. The enzyme concentration is 14000 U/g. The hydrolysis was conducted at pH 10.5 (SL1-PHS-3B pH-meter, Wuhan Midwest Instrument Co. Ltd., Wuhan, China) in a water bath shaker (New Brunswick Scientifics C24, Jintan, China). After hydrolysis, the enzyme was inactivated by placing the glass-stoppered bottle which contained the enzyme and the substrate in boiling water for 15 min. The hydrolysates were centrifuged using a GL-21 M refrigerated centrifuge (Xiangyi Instrument Co. Ltd, Changsha, China) at 5000 g for 20 min and the supernatants were then lyophilized (R2L-100KPS, Kyowa Vacuum Engineering, Tokyo, Japan) and stored in a desiccator for further use.

Hydroxyl radical scavenging activity assay

Hydroxyl radical scavenging activity was assayed using a modification of the method described by Li *et al* [16]. A mix of 600 μ L of 1,10-phenanthroline (5.0 mM), 600 μ L of FeSO₄ (5.0 mM) and 600 μ L of ethylenediaminetetraacetic acid (EDTA) (15 mM) were mixed with 400 μ L of sodium phosphate buffer (0.2 M, pH 7.4). Then 600 μ L of GI digests (2.0 mg/mL) and 800 μ L of H₂O₂ (0.01%) were added. The mixture was incubated at 37 °C for 60 min, and the absorbance was measured at 536 nm (UV754, Xianjian Scientific Instrument Co., Shanghai, China). Hydroxyl radical scavenging activity (H) was determined as in Eq 1.

$$H (\%) = (A_s - A_0) \times 100 / (A_c - A_0) \dots\dots\dots (1)$$

where A_s is the absorbance of the sample; A₀ is the absorbance of the blank solution using distilled water instead of sample; A_c is the absorbance of a control solution in the absence of H₂O₂.

DPPH radical scavenging activity assay

DPPH radical-scavenging activity was determined by the method of You *et al* [7]. The dried GI digests were dissolved in distilled water at 2.0 mg/mL. Thereafter, 2.0 mL of sample was mixed with 2.0 mL of 0.15 mM DPPH dissolved in 95 % ethanol. The mixture was then shaken vigorously a mixer (QT-1 Mixer, Tianchen Technological Co. Ltd, Shanghai, China) and kept for 30 min in the dark. The absorbance of the resulting solution was recorded spectrophotometrically at 517 nm and scavenging activity (S) was calculated as in Eq 2.

$$S (\%) = \{(A_s - A_c) / A_b\} 100 \dots\dots\dots (2)$$

where A_s is the absorbance of DPPH sample (i.e., 2 mL of sample solution mixed with DPPH solution); A_c is the absorbance of control (i.e., 2 mL of sample solution mixed with 2 mL of 95 % ethanol); and A_b is the absorbance of blank for DPPH (i.e., 2 mL of 95 % ethanol mixed with DPPH solution).

Reducing power assay

The reducing power was determined as previously described [17] with a slight modification. Briefly, 2.0 ml of sample was mixed with 2.0 ml of 0.2 M sodium phosphate buffer (pH = 6.6) and 2.0 ml of 1 % (w/v) potassium ferricyanide. The mixture was then incubated at 50 °C for 20 min. Then, 2.0 ml of 10 % trichloroacetic acid were added. After

centrifugation at 3000 r/min for 10 min, 2.0 ml of the supernatant were collected and mixed with 2.0 ml of distilled water and 0.4 ml of 0.1 % (w/v) FeCl₃. After standing at room temperature for 10 min, the absorbance was measured spectrophotometrically at 700 nm. Higher absorbance indicated stronger reducing power. An equivalent volume of distilled water instead of the sample was used as the blank.

In vitro pepsin–pancreatin simulated GI digestion

The GI digestion (an *in vitro* pepsin–pancreatin hydrolysis) was simulated according to the published methods with slight modifications [18]. CMPHs were re-dissolved (3.5 % w/v) in a buffer solution of 0.1 M KCl–HCl, and adjusted the pH to 2.0 with 1 M HCl. Then pepsin (4 % enzyme/substrate, w/w) was added. The mixture was incubated at 37 °C for 2 h, after which it was adjusted to pH 5.3 with a solution of 0.9 M NaHCO₃ solution and further to pH 7.5 with 1.0 M NaOH. Pancreatin (4 % enzyme/substrate, w/w) was added, and the mixture was further incubated at 37 °C for 2 h. In due time, the test tubes were kept in boiling water for 10 min to terminate the reaction. The GI digests were cooled down to room temperature and centrifuged at 11000 g for 15 min. The supernatant was lyophilized, and stored at –20 °C for further analysis. The test was carried out in triplicate. To investigate the changes in antioxidative activity of CMPHs digests during the simulated GI digestion, aliquots of GI digests were removed at 0 (Alcalase hydrolysate), 0.5, 1.0, 1.5, 2.0, (switch from pepsin to pancreatin) 2.5, 3.0, 3.5 and 4.0 during the digestion *in vitro*.

Amino acid analysis

The digest samples were precipitated with 10 % cold trichloroacetic acid for 2 h and then centrifuged at 11000 g for 15 min. The pH of the supernatant was adjusted to 2.0, and the solution was passed through a membrane (0.22 μ m). Then the filtrate was subjected to RP-HPLC analysis (Agilent HP1100) after precolumn derivatizing with PITC as indicated above to determine the free amino acid compositions according to the method of Zhu *et al* [18].

Determination of molecular weight distribution

The MW distribution of the digests was determined by gel permeation chromatography on a Superdex Peptide HR 10/300 GL column (10 × 300 mm, Amersham Biosciences Co., Piscataway, NJ, USA) with a UV detector at 214

and 280 nm. The mobile phase (isocratic elution) was 0.02 M sodium phosphate buffer containing 0.25 M NaCl (pH 7.2), at a flow rate of 0.5 mL/min. Six standard molecular markers, cytochrome C (12,500 Da), aprotinin (6500 Da), vitamin B12 (1355 Da), oxidized glutathione (612 Da) and glycylglycylglycine (189 Da) (Sigma Co., St. Louis, MO, USA), were used to calculate MW of the digests. UNICORN 5.0 software (Amersham Biosciences Co., Piscataway, NJ, USA) was used to analyze the chromatographic data.

Statistical analysis

All the experiments were carried out in triplicate and values expressed as mean \pm (standard deviation (SD)). The results were subjected to one-way analysis of variance (ANOVA). Duncan's new multiple range test was performed to determine significant difference between samples at 95 % confidence interval, using SPSS 13.0 software (SPSS Inc, Chicago, IL, USA).

RESULTS

The DPPH, hydroxyl radical scavenging abilities and reducing power were used as the standards to assess the antioxidative activity of CMPHs after *in vitro* gastrointestinal digestion and the results are shown in Fig 1.

DPPH radical-scavenging activity

After pepsin digestion, the DPPH radical-scavenging activity of CMPHs increased from 83.07 ± 1.57 to 88.45 ± 1.07 % (shown in Fig

1a). This same trend was also reported by You et al. Specifically, more hydrophobic amino residue side chain groups were expected to be exposed, which would make the peptides more accessible to the DPPH radicals and allow them to trap the radicals more easily during the pepsin digestion [7]. A sharp decrease ($p < 0.05$) of DPPH radical scavenging activity from 88.45 ± 1.07 to 40.69 ± 0.76 % was observed after 0.5 h pancreatin digestion (2.5 h). Pancreatin digestion for 4 h brought a markedly decrease in inhibition (more than 50 % of the antioxidative activity lost), which was in accord with the previous study made by Zhu et al, who confirmed that the increasing polarity of the pancreatin digest makes it more difficult to react with the lipid-soluble DPPH radicals [18].

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of CMPHs during the simulated GI digestion were shown in Fig 1a, which decreased significantly upon pepsin digestion ($p < 0.05$). Especially, after 0.5 h of digestion by pepsin, the hydroxyl radical activity of the GI digest was dramatically reduced by 23.2 % ($p < 0.05$) while further incubation with pepsin did not bring any further significant changes of activity. However, further digestion by pancreatin brought the hydroxyl radical scavenging activity of the final GI digest to 90.42 ± 1.28 % (i.e., 11.8 % increase compared to blank) ($p < 0.05$). The changing tendency of the hydroxyl radical scavenging activity of GI digests was different from that of the DPPH radical scavenging activity. That is because, after *in vitro*

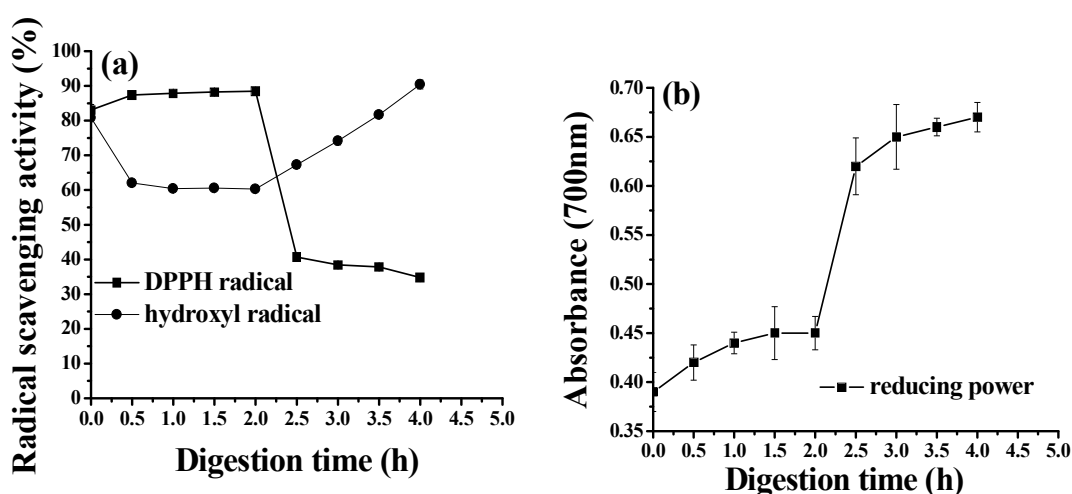


Fig 1: Changes in hydroxyl (a), DPPH (a) radical scavenging activity and reducing power (b) of CMPHs during sequential *in vitro* digestion. The data with different lowercase letters in the same test are significantly ($p < 0.05$) different

digesting, coconut meat peptides can be more effective hydrogen or electron donors. As a result, the antioxidative efficacy of CMPHs would approximately be 100 %, suggesting that new peptides with antioxidative activities were formed during GI digestion, and might increase the antioxidative efficacy.

Reducing power

The reducing power assay is used to evaluate the antioxidative activity based on its ability to reduce Fe^{3+} /ferricyanide complex to the ferrous form through the donation of an electron. As shown in Fig. 6b, the reducing power of the blank was 0.39 ± 0.02 %. It increased by 15.38 % compared to blank during the pepsin digestion. However, it increased significantly after 2 h of pancreatin digestion ($p < 0.05$). The final GI digest showed 71.8 % increase in reducing power compared to the GI digest at 0 h, which was in accordance with the report of Zhu *et al*, who also found an increased reducing power of zein hydrolyzates [18].

Amino acid composition and molecular weight distribution of peptides in digests

As shown in Table 1, two-step digestion process substantially increased the content of free amino acids (FAAs) of CMPH. Two-hour pepsin

digestion raised the FAA content of CMPH by 30.3 % compared to the blank. After further 2 h digestion with pancreatin, FAA of CMPH increased drastically by 86.7 % compared to the blank.

The molecular weight profiles show some differences between the gastrointestinal digested CMPHs and control (undigested) (shown in Table 2). During the first 2 h pepsin hydrolysis, the proportion of > 3000 Da fraction was reduced by 39.2 %; in particular, the > 5000 Da fraction was degraded significantly ($p < 0.05$). In addition, the 1000 Da to 3000 Da fraction range was reduced by 25.8 % was ($p < 0.05$). By contrast, the oligopeptides < 250 Da increased by 91.2 % ($p < 0.05$). However, the relative content of 250–1000 Da peptides just increased by 15.2 % ($p < 0.05$). During the following simulated intestinal digestion by pancreatin, the proportion of > 3000 Da fraction were further digested ($p < 0.05$), the proportion of 1000 to 3000 Da fraction was 55.8 % less ($p < 0.05$). The fraction of 250 - 1000 Da peptides were slightly increased ($p > 0.05$), wherein the oligopeptides < 250 Da increased by 97.8 % ($p < 0.05$), compared to those of the 2 h digest using pepsin. These changes are in accordance with that of the free amino acids described above. Moreover, the final GI digests were rich in < 500 Da fractions (about 65.2 %).

Table 1: Changes in free amino acid content of CMPHs during sequential *in vitro* digestion

Amino acid	Undigested (mg/g protein)	After gastric digestion (mg amino acid/g GI digest powder)	After intestinal digestion (mg amino acid/g GI digest powder)
Asp	3.8	4.3	6.8
Glu	25.6	26.7	28.2
Ser	5.8	7.6	11.5
Gly	4.2	4.9	7.1
His	4.5	7.9	17.5
Thr	3.6	4.2	6.4
Ala	8.4	9.1	10.3
Pro	5.6	5.9	7.5
Arg	24.2	25.4	36.4
Tyr	3.4	5.3	9.6
Val	12.1	13.7	19
Met	7.7	10.3	20.4
Cys	1.9	1.9	2.1
Ile	6.7	8.2	13.4
Leu	16.5	28.4	37.3
Phe	19.9	37.5	40.1
Lys	17.4	21.7	42.5
Trp	1.4	2	6.4
Total	172.7	225	322.5

Table 2: Protein fractions and their molecular weight of GI digests

Molecular weight (Da)	Undigested*(%)	After gastric digestion*(%)	After intestinal digestion*(%)
>5000	6.17±1.22 ^a	2.34±0.43 ^b	0.58±0.14 ^c
3000-5000	13.1±1.61 ^a	9.37±0.97 ^b	3.6±0.47 ^{ab}
1000-3000	30.33±1.16 ^a	22.51±1.18 ^a	9.94±0.84 ^b
500-1000	17.74±1.51 ^a	20.41±2.68 ^a	20.67±1.41 ^a
250-500	22.51±1.93 ^c	25.96±1.77 ^a	26.81±1.59 ^a
<250	10.15±1.2 ^a	19.41±2.21 ^a	38.35±2.17 ^a

*Undigested, after gastric digestion, after intestinal digestion, respectively, represent before digestion (0 h), after 2 h digestion by pepsin, and after following 4 h digestion by pancreatin; ^{a, b, c} data for each molecular weight range of CMPHs at three digestion time points were subjected to statistical analysis. Values with the same lowercase letters are not significantly different ($p > 0.05$)

DISCUSSION

Based on the above results obtained, it can be concluded that the overall antioxidant activities of CMPHs remained consistently high throughout *in vitro* digestion. The antioxidative activity of peptides is related to the composition and structural arrangement of amino acids, and influences their bioactivity and digestion stability. The amino acids analysis further supports the idea that the CMPHs were mainly broken into small fragments during pepsin digestion. However, during pancreatin digestion, these peptides were more completely hydrolyzed. Tyr, Met, His, Lys and Arg have generally been considered as antioxidative amino acids [19,20]. As shown in Table 1, the content of the above five free amino acids in GI digests increased from 53.8 to 126.4 mg/g. These antioxidative amino acids could act as proton/ electron donors to quench the free radical and promote the antioxidative activity of bioactive peptides, resulting in enhanced radical-scavenging activity and reducing power during digestion. The same findings were noted for peptides isolated from soybean hydrolysate [21] and casein-derived antioxidant peptides [22]. During *in vitro* digestion, CMPHs are cleaved to small peptides and free amino acids by pepsin and pancreatin. The molecular weight distribution of GI digests further confirmed that CMPHs was significantly degraded and produced more small oligopeptides and free amino acids during the simulated GI digestion. The low molecular weight oligopeptides could allow a rapid absorption and a high potential bioactivity [23]. This could be the cause of the enhanced antioxidative activity of CMPHs after digestion. Therefore, coconut meat-derived oligopeptides below 500 Da exhibited the best initial and surviving DPPH, hydroxyl radical scavenging activity and reducing power, which in accordance with the results observed by Ajibola *et al* [24]. These antioxidant peptides were most likely to be stable in real digestion system after ingestion in both stomach and intestine, which

have high proteolytic activity under acidic and alkaline pH, respectively.

CONCLUSION

The findings of this study show that CMPHs is subject to simulated GI digestion, and more oligopeptides of < 500 Da are formed during intestinal digestion. The FAA of CMPH is considerably higher than that of blank after digestion. The antioxidative activity of CMPHs is only slightly influenced by pepsin hydrolysis, but further digestion by pancreatin enhances the activity. Thus, coconut meat peptides can be used as food additives and diet nutrients to resist digestive proteases.

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