Data article

Title: *ACE-inhibitory and antioxidant peptides from coconut cake albumin hydrolysates: Purification, identification and synthesis*

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Abstract

The data presented in this article are related to the research article entitled "Purification and identification of ACE-inhibitory and antioxidant peptides from coconut cake albumin hydrolysates". This article describes the Purification, identification and synthesis of ACE-inhibitory and antioxidant peptides from coconut cake albumin hydrolysate. The field data set is made publicly available to the potential re-use of coconut cake or other plants by-products.

Specifications Table

Subject area	*Physics, Chemistry, Biology*
More specific subject area	*Identification and bioactivity of food-derived peptides*
Type of data	*Table, text file, graph, figure*
How data was acquired	*Survey(a Varioskan Flash, Thermo Scientific, USA; a HPP-L3 high-pressure apparatus , Tianjin HuaTaiSenMiao Biotechnology Co. Ltd., China), Mass spectroscopy (a LC-MS/MS with a coupled Eksigent Nano LC, Eksigent Technologies, Dublin, CA, USA, and Thermo LTQ linear ion trap mass spectrometer, Thermo Fisher, San Jose, CA, USA), Peptides synthesized (a solid phase procedure with a Liberty microwave peptide synthesizer, Mathews, NC, USA; a Kromasil 100-5 C_{18} column (4.6×250 mm; particle size 5 μm)).*
Data format	*Raw, filtered*
Experimental factors	*CCA, albumin extraction from defatted coconut cake; CCAH, coconut cake albumin hydrolyzed by four different enzymes in the following sequence: alcalase, flavourzyme, pepsin and trypsin.*
Experimental features	*Bioactivity peptides was isolated and identified from coconut cake albumin hydrolysates, and their ACE-inhibitory and antioxidant activity, effect on intracellular endothelin-1 content and cell oxidative stress were measured.*
Data source location	*China*
Data accessibility	*The data are available with this article*

Value of the data

- *The data provide the potential re-use of coconut cake or other plants by-products.*
- *The data provide information on how to isolate and identify bioactive peptides from coconut cake albumin.*
- *This data allows other researchers to extend the statistical analyses*

Data

Development of nutraceuticals or functional foods would be a low-cost alternative to conventional synthetic therapeutics which always has possible harmful side effects. Food derived bioactive peptides ACE-inhibitory and antioxidant activity receiving more interest [1]. Though some bioactive peptides have been identified from coconut water and globulin, bioactive peptide from coconut cake albumin has not been reported, which limited the usage of coconut cake in functional food or other industries [2, 3]. Previous study found that coconut albumin demonstrated considerable antioxidant and ACE inhibition activity [4]. Thus we investigated the purification, identification and synthesis of ACE-inhibitory and antioxidant peptides from coconut albumin. Moreover, the inhibition modality on ACE, stability against gastrointestinal enzymes, effect on intracellular endothelin-1 content, and ability on protecting vascular endothelial cells from reactive oxygen species mediated damage were also measured.

The RP-HPLC chromatography profiles and mass spectra of the bioactive peptides are shown in Figures 1-6.

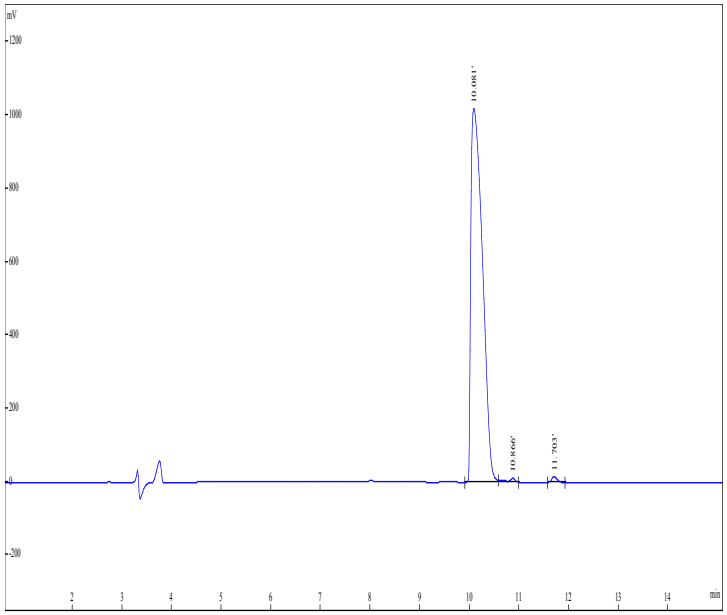

Fig. 1 The RP-HPLC chromatography profiles of identified peptide KAQYPYV

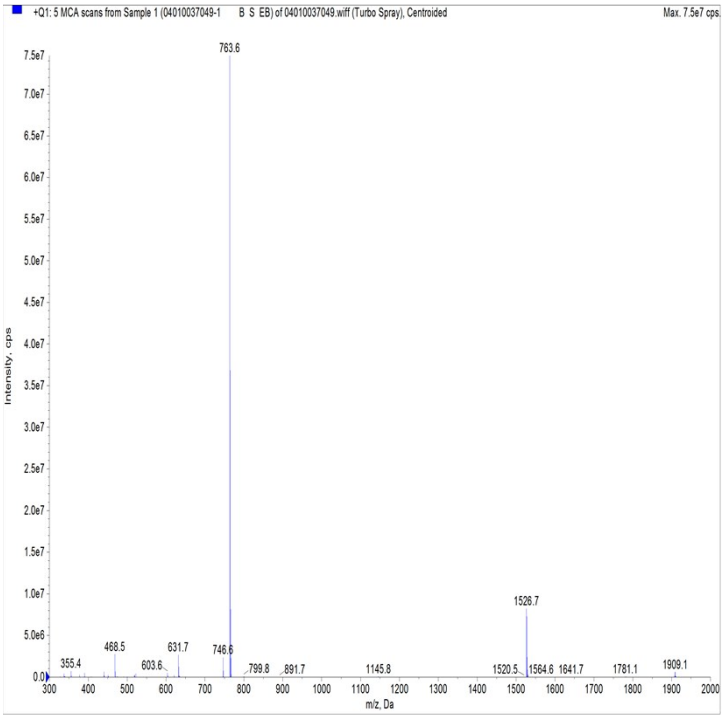

Fig. 2 The MS/MS analysis of identified peptide KAQYPYV

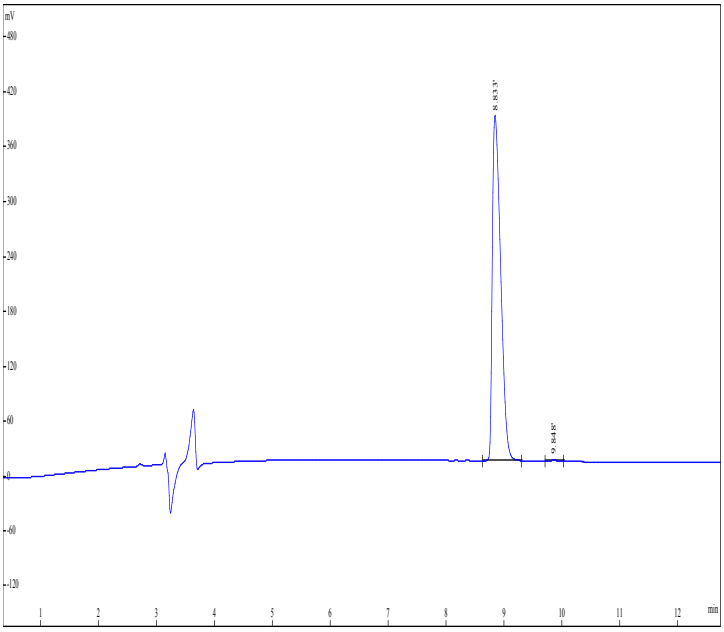

Fig. 3 The RP-HPLC chromatography profiles of KAQYPYV

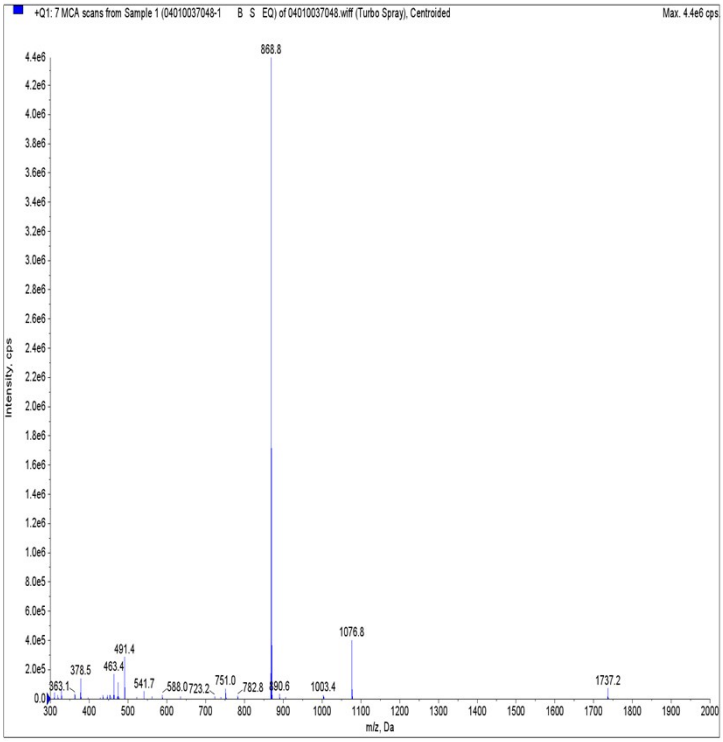

Fig. 4 MS/MS analysis of KIIIYN

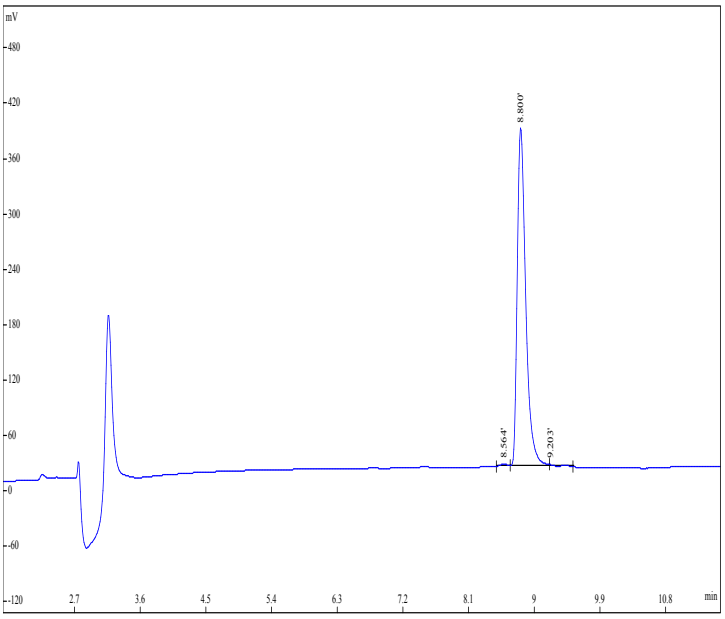

Fig. 5 The RP-HPLC chromatography profiles of KILIYG

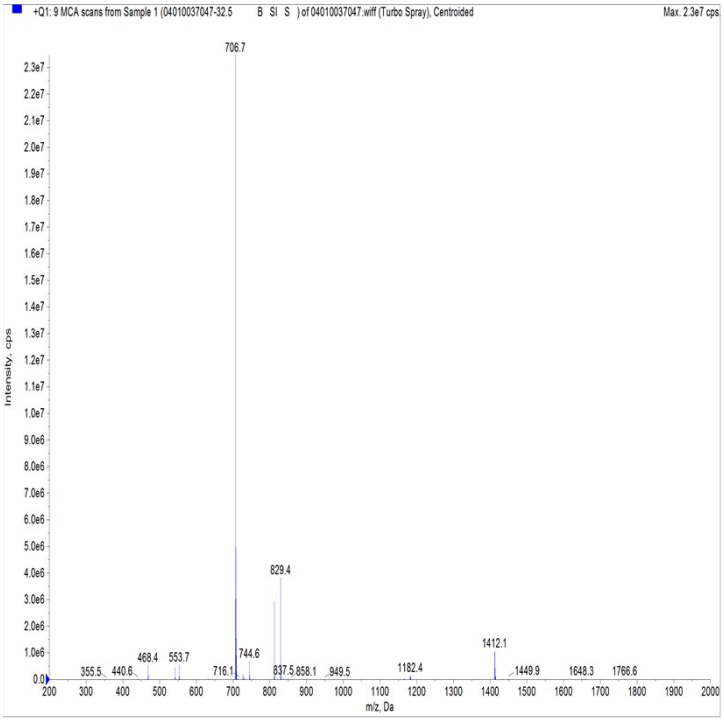

Fig. 6 MS/MS analysis of KILIYG

The data references to the purification, ACE-inhibitory activity, antioxidant ability, effect on intracellular endothelin-1 content, protection on vascular endothelial cells against oxidative damage, and stability against gastrointestinal enzymes can be seen in Table 1-8.

Table 1 Data on the Sephadex G-25 gel chromatography analysis of CCAH-III

Elution time (min)	Absorbance at 220 nm
5	0.001
10	0.001
15	0.001
20	0.001
25	0.001
30	0.005
35	0.017
40	0.256
45	0.879
50	0.81
55	0.632
60	0.876
65	1.378
70	1.262
75	1.074
80	0.895
85	0.872

90	1.045
95	2.603
100	2.588
105	2.422
110	1.228
115	1.042
120	0.916
125	0.808
130	0.74
135	0.688
140	0.676
145	0.645
150	0.544
155	0.356
160	0.184
165	0.11
170	0.075
175	0.069
180	0.065
185	0.058
190	0.048
195	0.04
200	0.035
205	0.032
210	0.029
215	0.026
220	0.024
225	0.025
230	0.026
235	0.032
240	0.039
245	0.048
250	0.056
255	0.061
260	0.062
265	0.06
270	0.057
275	0.039
280	0.031
285	0.023
290	0.019
295	0.016

300	0.015
305	0.014
310	0.012
315	0.01
320	0.009
325	0.007
330	0.006
335	0.005
340	0.006
345	0.004
350	0.004
355	0.004
360	0.004
365	0.004
370	0.004
375	0.004
380	0.004
385	0.004
390	0.004
395	0.004
400	0.004
405	0.007
410	0.017
415	0.011
420	0.009
425	0.01
430	0.012
435	0.012
440	0.012
445	0.012
450	0.011
455	0.011
460	0.01
465	0.005
470	0.004
475	0.004
480	0.004
485	0.004
490	0.004
495	0.004
500	0.004

Table 2 Data on the Sephadex G-25 gel chromatography analysis of Fraction A

Elution time (min)	Absorbance at 220 nm
5	0.003
10	0.003
15	0.003
20	0.005
25	0.004
30	0.013
35	0.005
40	0.253
45	1.232
50	1.481
55	1.14
60	0.853
65	0.727
70	0.614
75	0.545
80	0.589
85	0.613
90	0.522
95	0.458
100	0.451
105	0.466
110	0.477
115	0.438
120	0.544
125	0.78
130	1.811
135	1.218
140	0.329
145	0.309
150	0.408
155	0.508
160	0.516
165	0.434
170	0.301
175	0.187
180	0.115
185	0.088
190	0.061
195	0.052
200	0.049

205	0.039
210	0.039
215	0.036
220	0.037
225	0.061
230	0.074
235	0.102
240	0.141
245	0.172
250	0.187
255	0.194
260	0.174
265	0.141
270	0.109
275	0.07
280	0.044
285	0.025
290	0.018
295	0.01
300	0.011
305	0.012
310	0.01
315	0.007
320	0.018
325	0.016
330	0.017
335	0.019
340	0.012
345	0.017
350	0.013
355	0.023
360	0.017
365	0.006
370	0.007
375	0.01
380	0.004
385	0.006
390	0.026
395	0.016
400	0.027
405	0.019
410	0.014

415	0.021
420	0.013
425	0.012
430	0.023
435	0.021
440	0.02
445	0.029
450	0.027
455	0.03
460	0.029
465	0.02
470	0.026
475	0.019
480	0.021
485	0.021
490	0.021
495	0.022
500	0.015

Table 3 Data on the ACE inhibition and hydroxyl radical scavenging activity of CCAH fractions

Fractions	·OH scavenging activity Absorbance at 532 nm			ACE inhibition activity Absorbance at 228 nm		
control	0.083	0.083	0.083	1.539	1.54	1.433
CCAH	0.043	0.054	0.054	0.783	0.827	0.781
P1	0.065	0.073	0.076	1.633	1.676	1.745
P2	0.075	0.078	0.078	0.947	0.973	1.099
P3	0.071	0.068	0.072	1.051	1.047	1.175
VE	0.012	0.011	0.01	-	-	-
BHT	0.017	0.021	0.018	-	-	-
control	0.696	0.721	0.704	0.340276	0.3624346	0.349756
A1	0.344	0.355	0.295	0.220696	0.256806	0.204878
A2	0.294	0.307	0.266	0.100072	0.092696	0.119056
A3	0.279	0.229	0.306	0.171651	0.19113	0.172653
A4	0.152	0.117	0.098	0.060696	0.060306	0.077378
A5	0.242	0.209	0.198	0.222272	0.203269	0.194015
A6	0.152	0.181	0.183	0.090192	0.101562	0.113298
control	0.592	0.562	0.649	0.398737	0.424784	0.409844
A4a	0.084	0.065	0.085	0.086328	0.076663	0.096303
A4b	0.158	0.071	0.083	0.120554	0.143046	0.081364
A4c	0.296	0.311	0.277	0.233285	0.202592	0.189146
A4d	0.161	0.107	0.098	0.087778	0.103931	0.130912

A4e	0.088	0.051	0.054	0.051592	0.065778	0.051262
A4f	0.122	0.171	0.223	0.078792	0.085061	0.101216
A4g	0.106	0.096	0.127	0.182465	0.125903	0.146755
A4h	0.344	0.284	0.319	0.260563	0.227617	0.222179
A4i	0.123	0.121	0.197	0.066872	0.062695	0.119966
control	0.726	0.748	0.728	0.483489	0.469599	0.417666
A4e-1	0.268	0.244	0.183	0.153695	0.185409	0.127906
A4e-2	0.211	0.237	0.141	0.282649	0.299518	0.333693
A4e-3	0.044	0.084	0.059	0.050584	0.053257	0.070055

"-" in the Table meant not measured

Table 4 Data on the stability against gastrointestinal enzymes of the identified peptides

Peptides	Concentration (μg/mL)	·OH scavenging activity Absorbance at 532nm			Concentration (μg/mL)	ACE inhibition activity Absorbance at 228nm		
Control		0.546	0.583	0.622		1.220106	1.292511	1.189746
KAQYPYV	40	0.432	0.408	0.412	20	0.88957	0.902069	0.879336
	60	0.321	0.312	0.274	30	0.714705	0.660946	0.655298
	80	0.258	0.212	0.215	40	0.458702	0.450734	0.482001
	100	0.142	0.165	0.167	50	0.321167	0.325474	0.285477
	120	0.079	0.078	0.097	60	0.127333	0.128352	0.139218
Control		0.597	0.635	0.649		1.220106	1.292511	1.189746
KIIIYN	40	0.391	0.345	0.399	20	0.847992	0.758215	0.854685
	60	0.285	0.275	0.251	30	0.717006	0.760946	0.722524
	80	0.128	0.118	0.114	40	0.510616	0.498853	0.478894
	100	0.077	0.064	0.055	50	0.325418	0.2353987	0.355244
	120	0.035	0.044	0.047	60	0.168798	0.172404	0.148127
Control		0.625	0.645	0.599		1.220106	1.292511	1.189746
KILIYG	40	0.445	0.349	0.381	20	0.858833	0.809832	0.809837
	60	0.337	0.316	0.281	30	0.613402	0.655745	0.679703
	80	0.222	0.192	0.192	40	0.430348	0.469446	0.491038
	100	0.107	0.093	0.109	50	0.199198	0.224492	0.232894
	120	0.074	0.051	0.062	60	0.041529	0.049795	0.048389

Table 5 Data on protection of the identified peptides on EA.hy926 cells against damage induce by H_2O_2

Peptides	Concentration	Absorbance at 490 nm					
Blank		0.260276	0.2824346	0.269756	0.280276	0.2824346	0.279756
H_2O_2	300 μM	0.1428288	0.1555686	0.1249328	0.1365688	0.1345328	0.1294808
KAQYPYV	1.0 mg/mL	0.1779511	0.1843683	0.1856852	0.1896438	0.1763766	0.1817581
	1.5 mg/mL	0.2133292	0.2114304	0.1978918	0.2223292	0.1992182	0.2044804
	2.0 mg/mL	0.2217424	0.2170194	0.2026042	0.2190016	0.2190016	0.2096194
KIIIYN	1.0 mg/mL	0.1721683	0.1656851	0.1779511	0.1643683	0.1756852	0.1740511
	1.5 mg/mL	0.2091096	0.2006037	0.1999778	0.2053032	0.1895738	0.1951647
	2.0 mg/mL	0.2033292	0.2014304	0.1878918	0.2123292	0.2192182	0.2044804
KILIYG	1.0 mg/mL	0.1541518	0.155339	0.1538658	0.1483658	0.1431518	0.1653303
	1.5 mg/mL	0.1885456	0.1726633	0.1717545	0.1670633	0.1741634	0.1741637
	2.0 mg/mL	0.2032348	0.2050555	0.1964348	0.2035876	0.1899876	0.1975528
Captopril	1.0 mg/mL	0.2534414	0.2606581	0.2487011	0.2641801	0.2538023	0.2566712

Table 6 Data on superoxide radical scavenging activity of the identified peptides

Time (min)	Absorbance at 320 nm														
	Control			KAQYPYV			KIIIYN			KILIYG			Glutathione		
0	0.041	0.041	0.041	0.059	0.069	0.064	0.06	0.056	0.059	0.057	0.067	0.08	0.068	0.066	0.041
0.5	0.071	0.072	0.071	0.091	0.093	0.092	0.094	0.086	0.089	0.088	0.098	0.108	0.099	0.095	0.069
1	0.099	0.102	0.099	0.121	0.124	0.121	0.125	0.115	0.118	0.119	0.129	0.136	0.128	0.125	0.097
1.5	0.127	0.131	0.127	0.149	0.154	0.149	0.157	0.143	0.145	0.148	0.158	0.164	0.156	0.151	0.124
2	0.154	0.16	0.154	0.177	0.182	0.177	0.186	0.169	0.172	0.175	0.186	0.191	0.182	0.178	0.150
2.5	0.179	0.185	0.179	0.203	0.210	0.200	0.214	0.195	0.198	0.202	0.214	0.217	0.207	0.203	0.175
3	0.204	0.211	0.204	0.230	0.236	0.223	0.241	0.219	0.222	0.227	0.24	0.24	0.231	0.228	0.199
3.5	0.228	0.235	0.228	0.254	0.262	0.246	0.267	0.243	0.247	0.252	0.266	0.265	0.255	0.252	0.223
4	0.251	0.259	0.251	0.277	0.286	0.267	0.291	0.267	0.271	0.276	0.29	0.288	0.278	0.274	0.246
4.5	0.274	0.282	0.274	0.299	0.309	0.288	0.288	0.291	0.294	0.299	0.313	0.310	0.300	0.297	0.269
5	0.294	0.304	0.294	0.321	0.332	0.308	0.311	0.312	0.316	0.321	0.333	0.332	0.322	0.312	0.291

GSH (glutathione) was used as the positive control.

Table 7 Data on chelating ability of the identified peptides

Fractions	Absorbance at 562 nm		
Control	0.667	0.611	0.700
KAQYPYV	0.257	0.289	0.221
KIIIYN	0.292	0.232	0.205
KILIYG	0.271	0.154	0.214
Glutathione	0.213	0.266	0.172

Table 8 Data on the effect of the identified peptides on intracellular endothelin-1

Peptides	Concentration	Absorbance at 450 nm		
Control		80.34	81.66	78.78
KAQYPYV	1.0 mg/mL	70.37	75.64	68.97
	1.5 mg/mL	58.09	60.77	65.07
	2.0 mg/mL	62.44	54.56	51.78
KIIIYN	1.0 mg/mL	76.77	82.34	77.95
	1.5 mg/mL	74.58	76.98	73.04
	2.0 mg/mL	70.86	73.88	64.52
KILIYG	1.0 mg/mL	78.86	72.88	71.52
	1.5 mg/mL	62.09	60.77	65.07
	2.0 mg/mL	62.98	64.66	56.57
Catopril	1.0 mg/mL	37.42	36.85	41.22

Experimental Design, Materials and Methods

In the current study, the purification, identification, synthesis, stability and bioactivity of ACE-inhibitory and antioxidant peptides from coconut albumin were studied. Firstly, coconut cake albumin hydrolysates were prepared, and then ACE-inhibitory and antioxidant peptides were isolated and identified from it by sequential ultrafiltration, Sephadex gel chromatography, RP-HPLC and LC-MS/MS. The obtained peptides were synthesized and their stability against gastrointestinal enzymes, effect on intracellular endothelin-1 content, and ability on protecting vascular endothelial cells from reactive oxygen species mediated damage were also measured.

The material used in this study was coconut cake obtained from South coconut food Co., Haikou, China.

The used methods were as follows:

1. Extraction of coconut cake albumin (CCA)

Coconut cake was defatted three times with n-hexane (1:10, g/mL) and dried, ground and passed through a sieve of 0.2 mm mesh. The obtained defatted coconut cake were mixed with distilled water (1:15, g/mL), stirred at 4 °C for 1 h and then the mixture was filtrated. The filtrate was collected and the residue was resuspended in distilled water and stirred at 4 °C for 1 h. This step was repeated in three times. The filtrate was pooled and centrifuged at 10000g for 30 min, the supernatant was collected,

dialyzed against distilled water for 24 h at 4 °C. Then the dialyzation was centrifuged at 10000g for 30 min, and the supernatant was collected and lyophilized. Then CCA was obtained and stored at -20 °C.

2. Hydrolysis of coconut cake albumin

The enzymatic hydrolysis of CCA was performed following the method reported by Zheng, Li, Zhang, Ruan, Zhang, & Zhang et al. [5]. The CCA solution (5 g/100 mL) was transferred into polyethylene bags, sealed under vacuum and subjected to high pressure treatment using a HPP-L3 high-pressure apparatus (Tianjin HuaTaiSenMiao Biotechnology Co. Ltd., China) at 400 MPa, 35 °C for 15 min. Then the CCA solution was hydrolyzed by four different enzymes in the following sequence: alcalase (0.5 g/ 100g protein, 45°C, pH8.5, hydrolyzed time: 2 h), flavourzyme (0.5 g/ 100g protein, 50°C, pH7.0, 2 h), pepsin (0.3 g/ 100g protein, 37°C, pH2.0, 1 h), trypsin (0.3 g/ 100g protein, 37°C, pH7.0, 1 h). Before the addition of each enzyme, the temperature and pH of reaction solution were adjusted to the values described above. At the end of the hydrolysis, the reaction solutions were incubated in 100 °C for 5 min to inactivate the enzyme, and then centrifuged at 10000g, 4 °C for 20 min. The supernatants were collected and lyophilized to obtain CCAH. The degree of hydrolysis (DH), ACE-inhibitory ability and hydroxyl radical (·OH) scavenging activity of coconut cake albumin hydrolysis (CCAH) were determined using the methods of Adler-Nissen [6], Jimsheena & Gowda [7] and the 2-deoxyribose oxidation method [8], respectively.

3. ACE-inhibitory activity and inhibition pattern

According to the method of Jimsheena & Gowda [7], 50 µL of sample solution, 50 µL of ACE (25 mill units/mL) and 150 µL of 8.3 mM HHL were mixed and incubated at 37 °C for 60 min, followed by termination of the reaction by adding 250 µL of 1 M HCl. Then 1.4 mL of ethyl acetate was added and the mixture was vortexed for 5 sec and centrifuged at 14100g for 5 min. One mL of the upper organic phase was transferred into a test tube and placed in a vacuum oven at 80 °C for 1 h. Subsequently, 2 mL of distilled water was added and the absorbance at 228 nm was read. The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity. The inhibition (%) was calculated as below:

$$ACE\ inhibition\ (\%) = \left(1 - A_S/A_C\right) \times 100 \qquad (1)$$

Where, A_C was the absorbance of the ACE solution without an inhibitor, A_S was the absorbance of mixture contained samples. Determination of the ACE inhibition pattern was carried out according to the method published by Bush, Henry, & Slusarchyk [9]. Different concentrations of ACE-inhibitory peptide (0.1, 0.2, 0.5 mg/mL) were added to each reaction mixture and the enzyme activity was measured with different concentrations of HHL (0.76, 1.52, 3.04, 3.80 and 7.60 mM). The ACE-inhibitory activity was determined and the kinetics of ACE in the presence of the inhibitor was determined by Lineweaver-Burk plots.

4. Scavenging activity of hydroxyl radical

Hydroxyl radical (·OH) scavenging activity was assayed using the 2-deoxyribose oxidation method [8]. 100 µL of CCAH solution was mixed with 1.4 mL of sodium phosphate buffer (0.1 M, pH 7.4), 100 µL of 10

mM 2-deoxyribose, 100 µL of 10 mM FeSO4, 100 µL of 10 mM ethylenediaminetetra acetic acid (EDTA) and 100 µL of H_2O_2 (10 mM). After incubation at 37 °C for 1 h, 1.0 mL of trichloroacetic acid (28 mg/mL) and 1.0 mL of 2-thiobarbituric acid (10 mg/mL) were added. Then the mixture was kept in boiling water bath for 20 min. After cooled to room temperature, the absorbance of the mixture at 532 nm was read. The activity was determined using the equation:

$$\cdot OH \text{ scavenging activity } (\%) = [\,1 - (A_S - A_B)/A_C\,] \times 100 \qquad (2)$$

Where A_B was the absorbance of the blank (distilled water instead of samples), A_C was the absorbance of control (without the addition of 2-deoxyribose oxidation) and A_S was the absorbance of mixture contained samples. IC_{50} was defined as the concentration of peptide that was required to scavenge 50% of radical activity.

5. Purification of the bioactivity peptides

CCAH was separated by four steps in the following sequence: ultrafiltration, Sephadex G-25 gel chromatography, Sephadex G-15 gel chromatography and reversed-phase high performance liquid chromatography (RP-HPLC). Firstly, the CCAH was filtered sequentially using an ultrafiltration unit (Pellicon XL, Millipore, USA) through two ultrafiltration membranes with molecular weight (MW) cut-off of 5 and 3 kDa, respectively. Three fractions were obtained: CCAH-I with MW > 5 kDa, CCAH-II with MW 3–5 kDa and CCAH-III with MW < 3 kDa. The fraction with the highest ACE-inhibitory activity was further separated by a Sephadex G-25 gel filtration column (Φ2.6 cm × 60 cm) equilibrated with distilled water at 1.0 mL/min. The column was eluted with distilled water and monitored at 220 nm. Fractions were collected, lyophilized and their ACE-inhibitory activity and ·OH scavenging activity were measured. The fraction showing the highest ACE-inhibitory activity and/or ·OH scavenging activity was separated by Sephadex G-15 chromatography (Φ1.2 cm× 100 cm) equilibrated with distilled water at 0.8 mL/min. The column was eluted with distilled water and monitored at 220 nm. The fraction showing the highest ACE-inhibitory activity and/or ·OH scavenging activity was separated by RP-HPLC on a Zorbax semi-preparative C_{18} column (Φ 9.4 mm×250 mm, Agilent Technologies, USA), using a linear gradient of acetonitrile containing 0.1% TFA (5%-30%, in 30 min) at a flow rate of 2.5 mL/min. The fraction showing the highest ACE-inhibitory activity and/or ·OH scavenging activity was further isolated on a Zorbax analysis C_{18} column (Φ 4.6 mm×250 mm, Agilent Technologies, USA) with a linear gradient of acetonitrile containing 0.1% TFA (5%-25%, in 20 min) at a flow rate of 1.0 mL/min. The fractions with high ACE-inhibitory activity and/or antioxidant activity were rechromatographed to confirm their purity on the same analytical C_{18} column at a flow rate of 1.0 mL/min with a linear gradient of acetonitrile containing 0.1% TFA (15%–35%, in 12 min). The fractions with high ACE-inhibitory activity and/or ·OH scavenging activity were subjected to LC-MS/MS analysis.

6. Molecular mass and amino acid sequence of the purified peptides

Accurate amino acid sequence and molecular mass of the purified peptides were determined by LC-MS/MS with a coupled Eksigent Nano LC (Eksigent Technologies, Dublin, CA, USA) and Thermo LTQ linear ion trap mass spectrometer (Thermo Fisher, San Jose, CA, USA). The acquired MS/MS data were interpreted using the bioinformatics search engine Mascot version 2.1.0 (Matrix Sciences, London, UK).

The peptide sequences were matched to the published sequences of coconut proteins from the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) database.

7. Peptide synthesis

Bioactive peptides identified in CCAH were synthesized by the solid phase procedure with a Liberty microwave peptide synthesizer (Mathews, NC, USA). Briefly, the peptides were analyzed with a Kromasil 100-5 C_{18} column (4.6×250 mm; particle size 5 µm) eluted with mobile phase consisting of 0.1% TFA in water and 0.1% TFA in acetonitrile. The purity and molecular mass of the synthetic peptides were determined by Liquid Chromatography coupled to Mass Spectrometry.

8. Stability of the synthetic peptides

Peptide solutions were adjusted to pH 2.0, mixed with pepsin (E/S = 1:35) and incubated at 37 °C for 1 h. Afterwards, the reaction mixture was adjusted to pH 7.0, pancreatin was added (1 g/ 25g peptide) and incubated at 37 °C for 2 h, and then the digestion was terminated by boiling for 10 min. The ACE-inhibitory activity and ·OH scavenging activity of the treated synthetic peptides and the untreated synthetic peptides were determined.

9. Cell culture and cell viability determination

The EA.hy926 cells were seeded in a 96-well plate at a density of 1.0×10^5 cells mL^{-1}, cultured in DMEM (containing 1% non-essential amino acid solution, 10% FBS, 100 IU mL^{-1} penicillin and 100 µg mL^{-1} streptomycin) in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h. Then the wells were washed with phosphate buffer solution (PBS) and cultured in fresh DMEM but without FBS at 37 °C for 12 h. The DMEM without FBS was removed and the cells were cultured in DMEM at 37 °C for another 24 h. Then the cells were treated with different sample concentrations (1.0, 1.5 and 2.0 mg/ mL) for 24 h. Captopril (1 mg/mL) was used as the positive control, whereas the negative control was treated with only DMEM. Each treatment was repeated in six wells. After treatment, viability of the cells was measured using MTT method [10]. Briefly, the cells were washed with PBS and 20 µL of MTT (5 mg/ mL) were added into each well and kept at 37 °C for 4 h. Then MTT solution was removed and 100 µL of dimethyl sulfoxide were added. The absorbance was measured at 490 nm by a Varioskan Flash (Thermo Scientific, USA).

10. Effect on intracellular ET-1 content

The EA.hy926 cells were seeded in a 96-well plate at a density of 1.0×10^5 cells mL^{-1}. After being cultured in the DMEM at 37 °C for 24 h, the cells were treated with different concentrations of test samples (1.0, 1.5 and 2.0 mg/mL) for 48 h. Captopril (1 mg/mL) was used as positive control and the negative control was treated with only DMEM. After treatment, the growth medium of each well was collected to quantify ET-1 content using the ET-1 Elisa kit following the manufacturer's instructions.

11. Antioxidant activity of the peptides

11.1. Protective effect against H_2O_2-induced cellular oxidative stress

The EA.hy926 cells were seeded in 96-well plates with 5 × 10^3 cells mL^{-1} and incubated at 37 °C in 5% CO_2 for 24 h. Then the cells were treated with various concentrations of test samples (1.0, 1.5 and 2.0 mg/ mL, dissolved in PBS) and incubated at 37 °C for 24 h, followed by treatment with 300 μM H_2O_2 for another 24 h. Cell viability was determined using the MTT method as described above. In the blank control, cells were treated only with the DMEM. In the H_2O_2 treated group (control group), cells were treated with 300 μM H_2O_2. In the sample group, cells were pretreated with different concentrations of samples then exposed to 300 μM H_2O_2 [1]. The protective effect of samples against H_2O_2-induced cellular oxidative stress was calculated as follows:

$$Protective\ ability\ \% = (A_S - A_C)/\ (A_B - A_C) \times 100 \qquad (3)$$

Where, A_S was the absorbance at 490 nm of the sample group, A_C was absorbance of the H_2O_2 treated group, and A_B was the absorbance of blank control.

11.2. Superoxide radical-scavenging activity

According to the method of Marklund and Marklund [11], 100 μL samples (1 mg/mL) and 3 mL of pyrogallol solution (3 mM) were mixed and the absorbance at 320 nm recorded at 30 s intervals using a spectrophotometer. The scavenging activity was determined as the percentage of inhibiting pyrogallol autoxidation, which was calculated from the absorbance at 320 nm in the presence or absence of samples. GSH (1 mg/mL) was used as comparison.

11.3. Metal chelating capacity

According to the method of Jeong, De Lumen, & Jeong [12], 450 μL of sample solutions (100 μg/ mL), 45 μL of 2 mM $FeCl_2$ and 1815 μL of distilled water were mixed. The mixture was reacted with 90 μL of 5 mM ferrozine for 30 min. Then the absorbance was read at 562 nm. GSH (100 μg/ mL) was used as comparison and the chelating activity was calculated as follows:

$$Chelating\ activity\ (\%) = [\ 1 - (A_S - A_B)/A_C\ ] \times 100 \qquad (4)$$

Where A_B was the absorbance of the blank, A_C was the absorbance of control (without the addition of ferrozine) and A_S was the absorbance of mixture contained samples.

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