SUPPLEMENTARY MATERIAL

Extraction and composition characterization of amino acids from tung meal

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Abstract

The most desirable content of amino acids (AAs) in the extracted products from tung (*Aleurites fordii*) meal was 93.88%, which was obtained from shelled tung meal at a hydrolysis temperature of 45 °C and a isoelectric precipitation pH value of 4.4. Furthermore, the cytotoxic activity of extracted AAs was also evaluated by MTT assay. Antioxidant activity of extracted AAs was also measured by the DPPH· assay. As a result, the high yield of extracted AAs exhibited so low cytotoxic and high antioxidant activity that had the potential use as a functional ingredient.

Keywords: Amino acids; tung meal; cytotoxic activity; antioxidant activity

1. Experimental

1.1. Chemicals and reagents

Petroleum ether, ethanol, NaOH and 10% [w/v] HCl were purchased from Kelong Chemical Reagent Company (Chengdu, China) and were all analytical-grade. Bromocresol green, methyl red, ninhydrin, sodium citrate buffer solution (0.2 N, pH=2.2) and caustic potash were obtained from AstaTech Inc. (Chengdu, China). Caco-2 cells were purchased from West China School of Pharmacy (Sichuan, China). Fetal bovine serum (FBS), penicillin/streptomycin and *L*-glutamine were supplied by J&K Chemical Corp. (Beijing, China). 1,1-Diphenyl-2-picrylhydrazyl (DPPH·) was obtained from Sigma-Aldrich (Shanghai, China). Water was obtained from a Milli-Q Plus water purification system (Millipore, Bedford, MA, USA). Phosphate buffered saline was prepared in our laboratory. All other chemicals and reagents used were at analytical grade.

1.2. Sample preparation

1.2.1. Deoiling of tung meal

Tung meal was obtained from residual particles of tung seeds pressed tung oil by mould pressing. Tung seeds were collected from the fruits of tung trees (*Aleurites fordii*) yielded in east region of Sichuan, China. Shelled tung meal (STM) was defined as the residues of tung kernels, i.e. shelled tung seeds pressed tung oil. Unshelled tung meal (USTM) was defined as the residues of the same batch of tung seeds pressed tung oil.

Residual triglycerides of saturated and unsaturated fatty acids were removed from tung meal pressed tung oil. Firstly, 100 grams of STM was dissolved in 400 ml petroleum ether or ethanol under stirring. Then, the solution was heated and refluxed for 2 h. After cooling, residual triglycerides and solvent of STM were removed by filtration and rotary evaporation under vacuum. In the same way, deoiled USTM could be obtained and suited to the extraction of high purified AAs.

1.2.2. Extraction of AAs

AAs were extracted from deoiled tung meal by sequential hydrolysis from pH=10 to pH=5 and isoelectric precipitation in aqueous solution with different pH values. Firstly, deoiled STM or USTM was dissolved in distilled water under a high-speed homogenizer for 15min. Secondly, the pH value of the solution was adjusted to pH=10 by adding 10% [w/v] NaOH solution. The alkaline solution was heated to a certain

temperature and continuously stirred for 1 h. Then, tung meal was hydrolyzed in the aqueous solution with pH=5 for 0.5 h after neutralization resulting from 10% (w/v) HCl solution. Subsequently, the hydrolyzed solution was cooled and filtrated again so that the suspended substance in solution could be removed completely. Finally, the filtrated solution was kept in precipitating at the same hydrolysis temperature and a certain pH value adjusted by 10% [w/v] HCl solution for 24 h. In result, we could obtain large amounts of particles precipitated at the bottom of beaker gradually. After the process of vacuum freeze drying, the dried particles were collected for further study.

1.3. Method validation

The nitrogen content of tung meal and dried products during the hydrolyzing process was examined by Kjeltac 2200 Kjeldahl Nitrogen Analysis (KNA, FOSS, Sweden). The procedure of examination was that dried specimens were diluted, then NH_3 was distilled off, which was captured in a boric acid solution and the concentration was determined by titration with 0.1M HCl. The crude protein content was calculated by multiplying nitrogen percentage and the conversion factor (i.e. $\text{N} \times 6.25$).

The relative amino acid composition of tung meal and extracted products could be measured by L-8800 full-automatic Amino Acid Analysis (AAA, HITACHI, Japan). The samples were prepared in culture tubes containing sodium citrate buffer solution (0.2 N, pH=2.2) and performed in the analyzing column of ion exchange resin with a size of 4.6 × 60 mm at 135°C and a flow velocity of 0.4 ml/min. Then, separated substances were post-column derived by 3 pmol ninhydrin. AAs were quantificationally measured by fluorescence spectrometer at a wavelength of 570 nm and 440 nm, respectively. Glutamic acid was used as an internal standard.

The residual fatty acids in AAs were examined by Agilent 6890 with 5973 Gas Chromatograph-Mass Spectrometer (GC-MS, AGILENT, America). HP-5MS capillary column with a dimension of 30 m × 0.25 mm × 0.25 μm ($l \times d \times t$) was used as run chromatographic column. Helium gas was used as carrying gas at a constant flow rate of 1.5 ml/min. Samples were injected in a split mode of 10:1 and the electron impact ionization with 70 eV. The oven temperature was initially kept at 50°C for 2 min, then increased to 160°C at the rate of 30°C /min and held at 160°C for 15 min, increased to 280°C at the rate of 5°C /min and held at 280°C for 10 min, finally increased to 300°C at the rate of 30°C /min and held for 3 min.

1.4. Cytotoxic activity of AAs

To investigate the cytotoxicity of AAs extracted from STM and USTM by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, Caco-2 cells were selected as cultured cell of cytotoxicity assay (Malago et al. 2003).

Caco-2 cells were seeded in a 24-well cluster plates at a density of 4×10^4 cells/cm² and incubated for 24 h. The incubated medium was renewed and added in 100 μ L AAs cultured solution. The incubated cells were cultured for 48 h and replaced by fresh medium at interval of 4 h. The optical density (OD) of washed solution at different time was measured by EL-311 Bio-Tek microplate reader at $\lambda=570$ nm. The relative cell viability [%] in cluster plates was calculated according to the following equation:

$$\text{Cell viability [\%]} = \text{OD}_{\text{incubating}} / \text{OD}_{\text{reference}} \times 100$$

Where OD_{incubating} is the optical density of the incubated wells contained cultured cells and AAs extracted from STM and USTM, OD_{reference} is the optical density of the incubated wells contained only culture medium.

1.5. Antioxidant activity assay of AAs

The antioxidant activities of AAs extracted from STM and USTM were determined using DPPH $\cdot$ free radical scavenging assay measured on a UV-VIS-Spectrophotometer at 517nm. Stock solutions of DPPH in methanol, DPPH radical in ethyl acetate and model phenolic compounds in methanol (200 μ M) were prepared and stored in the dark at 25 $^{\circ}$ C.

A control consisted of 50 mL of 50% (v/v) methanol in 2.95 mL of DPPH $\cdot$ solution for AAs samples (Floegel et al., 2011). DPPH $\cdot$ radical scavenging capacity was calculated using following equation:

$$\text{DPPH}\cdot \text{ scavenging effects (\%)} = [1 - (A_1 - A_{1'}) / A_0 \times 100]$$

A₀ and A₁ correspond to the absorbance at 517 nm of the radical DPPH $\cdot$ in the absence and presence of sample solutions, respectively. A_{1'} is the absorbance at 517 nm of the radical DPPH $\cdot$ in the absence of DPPH solutions.

Additionally, hydroxyl radical scavenging activity of AAs extracted from STM and USTM was also determined and calculated using the following equation:

$$\text{Hydroxyl radical scavenging effects (\%)} = (A'_{1'} - A'_{0'}) / (A'_{1'} - A'_{0'}) \times 100$$

Where A'₁ is the absorbance at 520 nm of the presence of sample solution, A'_{1'} is the absorbance of the distilled water instead of H₂O₂, and A'₀ is the absorbance of the distilled water as black sample.

References

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Table S1. Percentage of AAs composition in samples extracted from STM and USTM at a temperature of 45°C and pH value of 4.4. AAs labeled “*” were represented as essential AAs.

Amino acids	Percentage of AAs [%]	
	In samples from STM	In samples from USTM
Asp	9.67	6.08
Glu	3.86	3.21
Ser	4.87	2.89
Thr (*)	15.66	10.49
Gly	4.58	3.67
Ala	4.34	3.05
Cys	0.23	0.11
Val (*)	7.73	6.87
Met (*)	1.94	1.26
Ile (*)	4.12	3.78
Leu (*)	7.77	6.84
Tyr	3.41	2.29
Phe (*)	5.28	4.87
Lys (*)	2.96	2.43
His	2.57	2.08
Arg	10.62	8.81
Pro	4.27	3.72
Total:	93.88	72.45

Figure captions:

Figure S1. Total AAs extracted from (a) STM and (b) USTM at a different temperature and pH value respectively.

Figure S2. Yield of hydrolyzed (a) STM and (b) USTM precipitated at a different temperature and pH value respectively.

Figure S3. GC-MS chromatogram of fatty acids in (a) STM and AAs extracted from STM, (b) USTM and AAs extracted from USTM.

Figure S4. Viability of Caco-2 cells incubated with AAs extracted from STM and USTM at a temperature of 45°C and pH value of 4.4. The measured time was performed at 4, 8, 16, 24, 32, 40 and 48 h, respectively.

Figure S5. The antioxidant activities of AAs extracted from STM and USTM by the absorbance of DPPH· free radical scavenging activity and hydroxyl radical scavenging activity.

Figure S1.

Figure S2.

Figure S3.

Figure S4.

Figure S5.