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Characterization of keratin microparticles from feather biomass with potent antioxidant and anticancer activities

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ABSTRACT

In the present study chicken feathers were hydrolyzed by chemical treatment in alkaline conditions. The pH value of feather hydrolyzed solution was amended accordingly the *iso*-electric precipitation. Two types of keratin microparticles KM1, KM2 were synthesized under acidic conditions at 3.5 and 5.5 pH respectively. The synthesized keratin microparticles possessed uniform and round surface by scanning electron microscopy (SEM). The thermal degradation of microparticles were examined by thermogravimetry (TGA). Fourier transform infrared spectroscopy (FTIR) revealed that the extracted keratin retained the most of protein backbone. The microparticles were screened for their *in vitro* anticancer activities by SRB bioassay towards HeLa, SK-OV-3 and A549 cancer cell lines. Futhermore, their cytotoxicity towards healthy cell lines was analyzed having Malin Darby canine kidney (MDCK) cell lines along with *in vitro* antioxidant activity using DPPH and ABTS methods KM1 and KM2 showed 200.31 ± 1.01 and 139.73 ± 0.94, 214.16 ± 0.29 and 153.92 ± 0.61, 328.92 ± 3.46 and 200.33 ± 2.48 μg/mL of IC_{50} levels against HeLa, SK-OV-3, and A549 cell lines, respectively. Moreover, KM1 and KM2 demonstrated significant antioxidant potency with IC_{50} levels 13.15 and 9.02 μg/mL as well as 8.96 and 5.60 μg/mL in DPPH and ABTS radical scavenging bioassay, respectively.

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1. Introduction

Feathers are a regular, renewable and natural stuff produced in abundance. Approximately 5 million tons of feather biomass is being generated annually from poultry farms [1,2] which leads to serious solid waste hazards [3]. The chicken feathers are the most troublesome waste product of the poultry industry [4]. Considering the high protein content (90%) [1], it could provide an excellent source of amino acids for animal feed [5], biocomposites [6] and for numerous other industrial applications [7,8]. Keratin is one of the most abundant biopolymers and is available from a variety of sources like hairs, nails, feather, wool, horn-hoof *etc.* [9–14]. Thus, due to serious environmental concern, these bio-based materials can be used to replace petrochemicals and can be applicable in chemical, pharmaceutical industries and tissue engineering too.

Keratin consists a chain of small amino acids with molecular weight approximately 10,500 Da [15]. It has α-helix, β sheet or random, unordered macrostructure. Particularly, keratin from chicken feathers mainly consist of β-sheet and little amount of α-helices and loops [16,17] and showed high percentage of amino acids such as alanine, glycine, serine, cystine and valine, but lower fraction of methionine, tryptophan and lysine [18]. The composition of amino acid may vary and depends on the source of keratin extracted [9]. Keratin is widely used in the pharmaceutical, cosmetic, medical, and biotechnological industry. It can be easily converted into porous foam of different shapes, sponges, microfibers coatings, mats, gels, and materials of high molecular weight.

Various techniques were used for extraction of keratin like reduction, oxidation and processing in ionic liquids [19–21]. Also, 2-mercaptoethanol was used as a thiol supplier which cleave the disulfide bonds without any alternation in the primary chain [22]. The sodium sulfide based extraction of keratin by dissolving chicken feather is an efficient and economically favorable method which provide sufficient yield and also retained the secondary structure of protein [23]. The feather protein also has potent

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antioxidant and anticancer properties. Recently the anti-cancerous activity of hair protein had been reported by [24].

$$Na_2S + H_2O \rightarrow 2Na^+ + HS^- + OH^-$$

Antioxidants play an important role in food processing and in human health. Thus, recovering the natural antioxidants from different bio-based products and natural proteins is a remarkable accomplishment [25,26]. Recently, various antioxidant peptides were reported and characterized from natural sources. The potential of feather hydrolysate as a strong antioxidant agent, has also been studied in literature in the last decade [18,27].

This study is aimed to assess the conversion of feather waste biomass into the biofunctional keratin microparticles. Thus, two kinds of bio-active protein precipitates were collected using *iso*-electric precipitation process and characterized for their antioxidant and anti-cancerous potential. The extracted keratin with different chemical properties can be used for numerous industrial applications.

2. Materials and methods

2.1. Materials

The chicken feathers were collected from a chicken processing plant at Jaya Gading, Kuantan, Malaysia. Sodium sulfide, NaOH, HCl, petroleum ether and Cetrimonium bromide (CTAB) were purchased from Sigma Aldrich (Selangore, Malaysia). Milli-Q water was used to make solutions and washing. 2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Dulbecco's Modified Eagle's Medium (DMEM) and RPMI-1640 medium supplemented with 10% of fetal bovine serum (FBS) and 1% Antibiotic–Antimycotic Solution (100X) were used and were purchased from Welgene (Daegu, Republic of Korea). Maintenance of all the cell lines was done in a humidified cell culture incubator with 5% of CO_2 at 37 °C temperature for cell growth. All chemicals were of analytical grade and used as received.

2.2. Pre-treatment of feathers

Wet feathers were cleaned and dehydrated in a ventilated oven at 40 °C for 72 h as described previously ASTM [28]. The degreasing of feathers was performed using petroleum ether for whole night, and followed by washing with double distilled water (dd H_2O). The washed feathers were conditioned at 20 °C and relative humidity (RH) 65% for 24 h. After first cleaning, feathers were treated with CTAB (g/L) for 3 h to remove the impurities and microbial contaminations. Those feathers were then cut into small pieces (2–25 cm), dried under sunlight for 48 h and stored at 4 °C for further usage.

2.3. Keratin extraction

25 g of the chopped feathers were immersed into sodium sulfide (0.5 M) [29] at 50 °C using mechanical stirrer for 6 h. The hydrolysate solution was filtered twice and centrifuged at 10,000 rpm to separate the supernatant. The solution pH was adjusted at different *iso*-electric points. During precipitation of the amino acids the particles were formed at pH value close to their *iso*-electric points [30,31]. The pH value of the hydrolysate solution was adjusted to pH 3.5 and 5.5 with 2N HCl, a thick layer of precipitates was settled down after 24 h. The precipitates were collected and marked as Keratin Microparticles (KM1 and KM2 respectively). In order to remove the salts and other impurities, the KM1 and KM2 were dispersed in water and centrifuged 3 times for 10 min at 12,000 rpm. Finally the keratin sediment was collected and lyophilized to obtain keratin powders. The total yield of extracted keratin powder was 79.6% and 70.23% for KM1 and KM2 respectively.

2.4. Surface morphology

The surface morphology of KM, KM2 microparticles was analyzed by scanning electron microscope (SEM) and confocal microscopy. It is performed by using Hitachi TM3030 Plus and Leica SP8 X respectively. In the preparation step of SEM, the samples were stacked directly onto an aluminum stub with a thin self adherent carbon film. The Fluorescein Isothiocyanate (FITC) tagged keratin microparticles were localized using confocal microscopy. FITC was dissolved in DMSO (2 mg/mL) and mixed with keratin (1 mg/mL) in 8.5 pH Tris-HCl buffer (50 mM). The whole solution was incubated at 150 rpm for 6 h in dark conditions. The excess FITC was removed by dialysis against dd H_2O. Fluorescently labeled keratin particles were stored under dark conditions. Laser scanning confocal microscope images were taken by Olympus confocal microscope (FV-1000).

2.5. TGA analysis

The Research Instruments TGA-Q 500 was used to perform thermo gravimetric measurements of microparticles under nitrogen atmosphere. The phase change temperature was measured with 10 °C and 900 °C range at heating rate of 10 °C/min. The vacuum drying of the samples was done at 40 °C. Approximately 3 mg samples were put on to aluminum crucible and the data was analyzed.

2.6. FTIR analysis

The microstructure and chemical characterization of microparticles were examined by using FTIR spectroscopy (Nicolet iS5 from Thermo scientific) in the transmission mode with the 4000 cm^{-1} and 500 cm^{-1} wave number range. It will help to detect the changes in chemical composition of peptides [32].

2.7. Nuclear magnetic resonance spectroscopy (NMR)

^{13}C NMR spectra of feather hydrolysate was recorded at 125 MHz respectively on a Ultrashield Advance III spectrometer. Sample was prepared in deuterated DMSO for NMR analysis. Chemical shifts on the δ scale are given in ppm, and having coupling constant in Hz. Chemical shifts were adjusted on the solvent peak [33].

2.8. DPPH free radical scavenging assay

The basis of the DPPH antioxidant bioassay is the reduction of the stable 2,2-diphenyl-1-picrylhydrazyl. The samples treated with DPPH, donate a proton which reduced diphenyl picrylhydrazine (non-radical) congener. The results of the DPPH bioassay are presented as a percentage of radical scavenging antioxidant (RSA%) activity of each sample. DPPH radical scavenging activity of the keratin microparticles KM1 and KM2 were calculated using previously given method [34,35]. The DPPH radical scavenging activity of ascorbic acid was used for comparison, and all tests were carried out in triplicate. The determination of RSA% results were according to Mensor et al. [36] as given in the equation:

$$\%\ \text{Scavenging} = \frac{1/4\ \text{Absorbance of blank} \times \text{Absorbance of test}}{\text{Absorbance of blank } 100} \times 100$$

A plot of test samples concentration and% scavenging introduced IC_{50} in the existence of an Ascorbic acid as standard.

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