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Preparation and characterization of hydroxypropyl chitosan modified with collagen peptide

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ABSTRACT

Collagen peptide (COP) had been grafted to hydroxypropyl chitosan (HPCS) by using microbial transglutaminase (MTGase) as biocatalyst. HPCS was synthesized from chitosan and propylene oxide under alkali condition. The chemical structures of derivative were characterized by FT-IR and ^{1}H NMR spectroscopy. The process conditions were optimized from the aspects of the reaction time, the reaction temperature, the molar ratio of COP and the mass ratio of MTGase to HPCS. In this study, HPCS-COP could serve not only to reduce the loss of moisture but also to absorb the moisture, and the moisture absorption and moisture retention abilities were closely related to the degree of substitution (DS) values. In addition, with the DS and concentration increase of HPCS-COP, the radical scavenging activity increased in vitro antioxidant activity. Furthermore, the methyl thiazolyl tetrazolium assay (MTT) was applied to evaluate the biocompatibility of HPCS-COP, and the result indicated that HPCS-COP with the DS of 0.34 displayed pronounced cell viability at 50 ppm. Therefore, the results suggest that HPCS-COP could be potential wound dressings for clinical applications.

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1. Introduction

Skin is a dynamic and integrated organ with the largest surface area in human body [1]. Its normal functions can be gradually decreased by many factors, such as aging, health status, environmental pollutants, and it can be affected by severe wounds including chronic ulcers and burns [2]. Effective wound dressing not only protects the wound from surrounding environments but also provides moist environment for continuous tissue reconstruction process [3]. Biomaterials have the advantage of forming part of the natural tissue matrix, and some play an active part in normal wound healing and new tissue formation [4], these biomaterials include collagen, hyaluronic acid, chitosan, alginates and elastin [5].

Chitosan, as a natural renewable resource, has a number of unique properties such as biocompatibility, biodegradability and non-toxicity [6]. Chitosan has prospective applications in many fields such as biomedicine, wastewater treatment, functional membranes, food, tissue regeneration and cosmetic or personal care [7]. The applications of chitosan are limited because of its poor solubility. Hydroxypropyl chitosan (HPCS) is an important derivative of chitosan, which allows specific chemical modifications for its primary amine groups, and this reactive site enables the grafting of a large variety of properly functionalized molecules [8]. Recently, more and more experimental results have been reported on the potential applications and exploitations of HPCS based materials in wound healing and skin burn [9].

Collagen is the most abundant protein in mammals where it serves as the primary component of many load-bearing tissues which include skin, ligaments, tendons, and bone [10]. Collagen based materials are biocompatible, biodegradable, and weakly antigenic [11]. Due to its low antigenicity and inherent biocompatibility with most endogenous tissue, collagen has often been used for surgical repair [12]. Collagen is also extensively utilized in the cosmetic industry as a water-retain and moisturizing agent. Collagen based wound dressings have long been used to cover burn wounds and treat ulcers [13]. Collagen peptide (COP) as a functional ingredient [14] is hydrolyzed from collagen, which is acknowledged as one of the most promising biomaterials in wound healing application [15].

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As a biocatalyst, microbial transglutaminase (MTGase) is an extracellular enzyme with a remarkable characteristic of Ca^{2+} independent catalytic property, furthermore, acts in a wide range of temperature and pH values [16]. The MTGase is used to catalyze macromolecular grafting and crosslinking of proteins [17]. MTGase catalyzes the formation of isopeptide between the group of γ-carboxamides (-C(O)NH2) of glutamine residues (donor) and primary amines or ε-amino group of lysine(acyl donor) [18]. MTGase has been shown to crosslink gelatin [11], collagen [18] and soy protein isolate [19] for tissue engineering and drug delivery [20].

In this study, MTGase was thought to catalyze the reaction between HPCS and collagen peptide (COP), and the reaction conditions were optimized. Then the ability of moisture absorption and retention was measured in different humidity. The scavenging effect on hydroxyl radical and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was used to evaluate the antioxidant activities of HPCS-COP. In addition, MTT assay evaluated the impact of HPCS-COP on proliferation of the NIH-3T3 mouse embryonic cells.

2. Materials and methods

2.1. Materials

Chitosan (CS) (Mw 520,000) with a 92% degree of deacetylation (DD) was supplied by the Golden-Bell (Cochin, India). Collagen peptide (Mw 800) was purchased from Sichuan Mingrang Biological Technology Co., Ltd., Sichuan, China, without further purification. Microbial transglutaminase (MTGase) were purchased from Huashun Biological Technology Co., Ltd., Wuhan, China. Sodium hydroxide, isopropyl alcohol, propylene epoxide, tetramethylammonium hydroxide, and other chemicals used in this investigation were analytical grade without further purification. They were purchased from Sinopharm Group Chemical Reagent Corp. All water used in this investigation was distilled and deionized.

2.2. Preparation of HPCS

HPCS was prepared based on the previous research with slight modification [21]. Briefly, chitosan (20 g) was alkalized by adding 20 ml 50% (w/w) NaOH aqueous solution, completely mixed, and kept at −18 °C for 24 h. The mixture was then thawed and transferred to a three-necked round-bottom flask contained 200 ml isopropyl alcohol. After vigorously stirring for 30 min at room temperature, 2 ml 25% tetramethylammonium hydroxide (TMAH) solution and 200 ml propylene oxide were added with stirring over 1 h and then refluxed for 5–6 h at 45 °C with continuous stirring. The resulting mixture was subsequently filtered and then dialyzed with the 8000–10,000 molecular weight cut-off dialysis tubing against distilled water for 3 days. Finally, the product was dried at 40 °C in vacuo for 24 h. And the DS of HPCS was determined according to the ^{1}H NMR.

2.3. Purification of MTGase

MTGase was first poured into 0.2 mol/L KH_2PO_4-NaOH buffer solution (pH 6.5). The liquid was centrifuged at the speed of 4000 rpm for 10 min, the supernatant was adjusted to pH 5.6 with 1.0 mol/L HCl, after removal of the insoluble fraction, the pH value of the supernatant was adjusted to 4.2 with 1.0 mol/L HCl, and then centrifuged. The supernatant was purified by dialysis through the 8000–10,000 molecular weight cut-off dialysis tubing for 3 days. The dialyzed MTGase was lyophilized for 24 h to obtain the MTGase lyophilized power.

2.4. Preparation of HPCS-COP

In a typical reaction procedure, the prepared HPCS (1 g) and COP (1 g) were respectively dissolved in 0.2 mol/L Na_2HPO_4/NaH_2PO_4 buffer solution (PBS, pH 6.0) to prepare aqueous solution and they were mixed together to form homogeneous solution. MTGase powder (0.2 g) was dissolved in PBS solution and added to the above mixture to catalyze the reaction between HPCS and COP. Magnetic stirring was continuous for 5 h at 40 °C. Then the solution was treated in boiling water for 10 min and later cooled to room temperature. The HPCS–COP solution was obtained after filtering with cup-type ultrafilter. Subsequently, the solution was neutralized with 20% (w/w) NaOH aqueous solution and then purified by dialysis through the 8000–10,000 molecular weight cut-off dialyses tubing for 3 days. The dialyzed product was finally freeze-dried to obtain the purified HPCS–COP. The dried products were stored in vacuum desiccators over P_2O_5 for further analysis. The procedure of synthesizing HPCS-COP was as follows in Scheme 1.

2.5. Optimization of reaction condition of preparing HPCS-COP

Four independent variables were investigated, including the reaction time (1, 2, 4, 5 and 6 h), the reaction temperature (30–70 °C), the mass ratio of COP to HPCS (0.8, 1.0, 1.2, 1.3 and 1.4) and the mass ratio of MTGase to HPCS (0.1, 0.15, 0.2, 0.25, and 0.3). Their variables were fixed at a certain value, while changing only one variable value.

2.6. Characterization of the derivatives

2.6.1. Fourier transforming infrared spectroscopy (FT-IR) analysis

FT-IR spectra of HPCS-COP, HPCS and CS samples were performed with a Nicolet 5700 Fourier transform infrared spectrometer (USA) in the wavenumber ranging from 400 to 4000 cm^{-1}. The samples were prepared by the KBr-disk method.

2.6.2. ^{1}H NMR characterization

The ^{1}H NMR spectra of CS and its derivatives were recorded on a Bruker AMX-500 NMR spectrometer at an ambient temperature. The samples were dissolved in 1%DCl D_2O and D_2O. Tetramethylsilane (TMS) was used as the internal standard.

2.6.3. Measurement of the degree of substitution

The degree of substitution (DS) is defined as the number of amine groups substituted per repeating structural unit of the HPCS. In this work, the DS was measured according to the method of Fan et al. [22], the concentration of COP between 0.001 g/l and 0.02 g/l was liner relation with absorbance at 200 nm by UV spectrophotometer. And the standard curve for the liner relation was described as Eq. (1). The HPCS and HPCS-COP were also measured with absorbance at 200 nm by ultraviolet spectrophotometry with the concentration of 0.02 g/l. The HPCS sample was the blank control. The DSs of HPCS-COP were determined by Eq. (2).

$$A = 43.97C + 0.17 \quad R^2 = 0.996 \tag{1}$$

$$DS = \frac{237C}{16 - 783C} \tag{2}$$

where A was the absorbance of COP, C was the concentration of COP.

2.7. Moisture absorption and retention test [23]

Prior to the moisture-absorption testing, the samples were dried over P_2O_5 in vacuo for 24 h. The water-absorption ability was eval-

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