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Transglutaminase-catalyzed grafting collagen on chitosan and its characterization

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ABSTRACT

Collagen grafted chitosan was prepared with microbial transglutaminase (MTGase) as biocatalyst which showed high efficiency, selectivity, mild reaction condition and environmental friendliness. The reaction conditions that influenced the degree of substitution (DS) were optimized, which included the reaction time, the reaction temperature, the mass ratio of collagen to chitosan and the mass ratio of MTGase to chitosan. In this study, the water-solubility collagen–chitosan 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 DS values. In addition, in vitro antioxidant activity was evaluated in terms of DS values and concentration. Furthermore, L929 mouse fibroblasts were cultured with collagen–chitosan, and methylthiazol tetrazolium (MTT) assay exhibited that collagen–chitosan with DS of 0.660 displayed pronounced cell viability at 2.5 mg/ml. Therefore, the water-soluble collagen–chitosan showed the potentiality to repair skin in cosmetic, biomedical and pharmaceutical fields.

1. Introduction

Skin is a dynamic and integrated organ with the largest surface area in human body. Its normal functions can be gradually decreased by many factors, such as aging, health status, environmental pollutants and wounds (Lee et al., 2012). UV irradiation and burns also disturb the balance between the formation of reactive oxygen species and the capacity of antioxidant system in several ways (Frisman, Racz, & Chmelarova, 2013). At high levels of oxidative stress, the significant imbalance between free radicals and antioxidant defense system can lead to cell injury (Lin et al., 2013). Over the past few decades, natural biomaterials such as collagen, silk and chitosan had received increasing attention in food, cosmetic, biomedical and pharmaceutical fields due to their unique properties, such as biodegradability, biocompatibility and non-toxicity (Aramwit, Siritientong, Kanokpanont, & Srichana, 2010; Li et al., 2013). It was also reported that some natural polysaccharides and peptides had the ability to promote cell attachment, proliferation and tissue regeneration (Chen, Wang, Chen, Ho, & Sheu, 2006; Koide, 2007; Ueno et al., 1999).

Collagens, characterized by a unique triple-helical structure, are the cardinal component of extracellular matrices existing in all multicellular animals (Koide, 2007). Collagen has favorable biodegradability, biocompatibility, antioxidant activity, reparative ability to skin, and weak immunogenic reactions (Fujii et al., 2013; Sangeetha, Ramamoorthy, Sreeram, & Nair, 2012; Sun et al., 2009). And collagen is extensively utilized in the cosmetic industry as a water-retain and moisturizing agent. Recently, the use of fish collagen is remarkable because of its high availability and it could reduce the potential for disease transmission (Shen et al., 2008). Therefore, fish scale collagen is acknowledged as one of the most promising biomaterials in wound healing application (Gopinath, Kumar, Selvaraj, & Jayakumar, 2005).

Chitosan is a natural hydrophilic biomacromolecule abundantly found in crustaceans (Muzzarelli et al., 2012). Structurally, it is the linear (1-4)-2-amino-2-deoxy-β-D-glucan, randomly acetylated to a minor extent (Francis Suha & Matthewb, 2000). As the only cationic polysaccharide in nature, chitosan has attracted the attention of researchers owing to biocompatibility, biodegradability, antimicrobial activity, and peculiar biological properties in wound healing (Muzzarelli, 2009). However, chitosan is normally insoluble in aqueous solution above pH 7 because of the stable, crystalline structure (Chen, Wang, Liu, & Park, 2002). So improving

Abbreviations: MTGase, Microbial transglutaminase; R_a, Moisture-absorption; R_h, Moisture-retention; DS, The degree of substitution; MTT, Methylthiazol tetrazolium; Vc, Ascorbic Acid; HA, Hyaluronic acid.

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the solubility of chitosan is the key step to exploit its application. PEG-grafting, hydroxylation, sulfonation, quaternarization and carboxymethylation are the common methods to modify chitosan. However, chemical crosslinking agents frequently induce toxic side effects (owing to residual agents) or to secondary reaction with unwanted products (Lin, Liang, Chung, Chen, & Sung, 2005). And compared with 1-ethyl-(dimethylaminopropyl) carbodiimide and *N*-hydroxy sulfosuccinimide crosslinking method (Fan et al., 2013), the enzyme-catalyzed reaction time is distinctly shortened and the degree of substitution (DS) of product is higher. Therefore, a great interest is devoted to focus on enzyme catalyzed reactions to modify the chitosan. Enzyme-catalyzed reactions show a variety of benefits, including the high efficiency, selectivity, mild reaction condition and environmental friendliness (Bertoni, Barbani, Giusti, & Ciardelli, 2006).

As a biocatalyst, microbial transglutaminase (MTGase) is an extracellular enzyme with a remarkable characteristic of calcium-independent catalytic property, furthermore, exhibits a wide substrate specificity being also active over a wide range of temperature and pH values (Porta, Mariniello, Pierro, Sorrentino, & Giosafatto 2011). The MTGase is used to catalyze macromolecular grafting and crosslinking of proteins (Wu, Bentley, & Payne, 2011). And it catalyses the formation of covalent linkages between γ-carboxyamide groups of peptide-bound glutamine residues and ϵ-amino groups of lysine or primary amino groups of a number components (Chen, Embree, Brown, Taylor, & Payne, 2003; Kołodziejska, Piotrowska, Bulge, & Tylingo, 2006). For the sake of integrating mutual advantages, researchers have tried to conjugate chitosan with variety of proteins or peptides (Sang, Zhou, Yun, & Zhang, 2010).

In this paper, collagen–chitosan was synthesized with MTGase as biocatalyst, and the reaction conditions were optimized. Then the ability of moisture absorption and retention was measured in different humidity. The antioxidant activity of collagen–chitosan was discussed in terms of the hydrogen peroxide scavenging activity and DPPH radical scavenging activity. In addition, the impact of collagen–chitosan on proliferation of L929 fibroblasts was investigated.

2. Materials and methods

2.1. Materials

Chitosan (Mw 520,000) with a 92% degree of deacetylation was supplied by the Golden-Bell (Cochin, India). Fish scale collagen (Mw 3000) was purchased from Sichuan Mingrang Biological Technology Co. Ltd., Sichuan, China, without further purification. Microbial transglutaminase (MTGase) from Streptoverticillium mobaraensis were purchased from Huashun Biological Technology Co. Ltd., Wuhan, China. Dubecco's Modified Eagle Medium (DMEM) was purchased from Thermo Co. Ltd, Beijing, China. Fetal bovine serum (FBS) from Sijiqing Co. Ltd., Zhejiang, China. Methylthiazol tetrazolium (MTT) was purchased from Sigma, Deisenhofen, Germany. Acetic acid, sodium hydroxide and other reagent used in this investigation were of analytical grade and without further purification. They were purchased from Sinopharm Group Chemical Reagent Corp.

2.2. Purification of MTGase

MTGase was first poured into 0.2 mol/l Na_2HPO_4/NaH_2PO_4 buffer solution (PBS, pH 5.0). The liquid was centrifuged at the speed of 3000 rpm for 10 min. Then the supernatant was purified by dialysis through the 8000–10,000 molecular weight cut-off dialysis tubing for three days. The dialyzed MTGase was lyophilized for 24 h to obtain the MTGase lyophilized power.

2.3. Preparation of collagen–chitosan

In a typical reaction procedure, chitosan (1 g) was dissolved in 1% acetic acid and the solution was adjusted to pH 6.0. Collagen (1 g) was dissolved in PBS (0.2 mol/L, pH 6.0). Then they were mixed together to form homogeneous solution. Thereafter, MTGase powder (0.1 g) was also dissolved in PBS solution, and then it was added to the above mixture to catalyze the reaction between chitosan and collagen. Magnetic stirring was continuous for 1 h at 40 °C. Then the solution was treated in boiling water for 10 min and later cooled to room temperature. The collagen–chitosan solution was obtained after filtering with qualitative filter paper. Subsequently, the solution was neutralized with 20% (w/w) aqueous NaOH and then purified by dialysis through the 8000–10,000 molecular weight cut-off dialysis tubing for three days. The dialyzed product was finally freeze-dried to obtain the purified collagen–chitosan. The dried products were stored in vacuum desiccators over P_2O_5 for further analysis.

2.4. One-factor-at-a-time experiment

Four independent variables were investigated, including the reaction time (0.5, 1–4 h), the reaction temperature (20–60 °C), the mass ratio of collagen to chitosan (0.6, 0.8, 1.0, 1.2 and 1.4) and the mass ratio of MTGase to chitosan (0.06, 0.10, 0.14, 0.18 and 0.22). Their variables were fixed at a certain value, while changing only one variable value (Lin et al., 2013).

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

FT-IR spectra of collagen–chitosan samples and chitosan 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. 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 chitosan. In this work, the DS was measured according to the method of Fan (Fan et al., 2013), the concentration of collagen between 0.001 g/l and 0.05 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 chitosan and collagen–chitosan were also measured with absorbance at 200 nm by ultraviolet spectrophotometry with the concentration of 0.05 g/l. The chitosan sample was the blank control. The DSs of collagen–chitosan were determined by Eq. (2).

$$\begin{aligned} A &= 34.05C + 0.030 \\ R^2 &= 0.994 \end{aligned} \quad (1)$$

$$\mathrm{DS} = \frac{1526.45C}{150 - 2999C} \quad (2)$$

where A is the absorbance of collagen, C is the concentration of collagen.

2.7. Water solubility testing (Mao, Shuai, Unger, Wittmar, Xie & Kissel, 2005)

Solubility of collagen–chitosan was measured at different pH values at room temperature. Briefly, the samples were dissolved in a 0.25% acetic acid solution (2 mg/mL), the pH value of the solution was adjusted using NaOH and the transmittance of the solution at