Reactive & Functional Polymers 76 (2014) 26–31

Contents lists available at ScienceDirect

Reactive & Functional Polymers

journal homepage: www.elsevier.com/locate/react

Enzymatic synthesis of collagen peptide–carboxymethylated chitosan copolymer and its characterization

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ARTICLE INFO

Article history:
Received 5 December 2013
Received in revised form 6 January 2014
Accepted 20 January 2014
Available online 27 January 2014

Keywords:
Collagen peptide
Microbial transglutaminase
Antioxidant activity
L929 fibroblast
Cell viability

ABSTRACT

Collagen peptide grafted carboxymethylated chitosan was synthesized by using microbial transglutaminase as bio-catalyst. The catalyzed reaction exhibited high efficiency, selectivity, environmental friendliness. Collagen peptide grafted carboxymethylated chitosan was characterized by Fourier transform infrared spectroscopy. The process conditions were optimized including the reaction time, the reaction temperature, the molar ratio of collagen peptide to carboxymethylated chitosan and the pH value. The different molecular weight, concentration and degree of substitution of collagen peptide–carboxymethylated chitosan had crucial effects on the hydrogen peroxide scavenging activity of collagen peptide–carboxymethylated chitosan. The methyl thiazolyl tetrazolium assay showed that at a suitable concentration collagen peptide–carboxymethylated chitosan with different degree of substitution value could promote L929 mouse fibroblasts effectively. Therefore, the results suggest that collagen peptide–carboxymethylated chitosan could be potential wound dressings for clinical applications.

1. Introduction

Burn has been a global public health problem that leads to millions of patients with disabilities and hospitalization per year [1]. It is important to protect wound and promote burn wound healing during the cure. Over the past few decades, it was reported that some natural polysaccharides and peptides had the ability to promote cell attachment, migration, proliferation and tissue regeneration, and they showed huge potential as the accelerator of wound healing in biological and pharmaceutical applications [2–5].

As natural polysaccharides, chitosan (CS) possesses many attractive properties such as biological activity, antibacterial activity, and anticancerous property [6–8]. However, it is suffering due to its poor water-solubility which largely restricts its application [6]. So it is essential to modify chitosan [3]. Among the water-soluble chitosan derivatives, carboxymethylated chitosan (CMC) in particular is an excellent bio-material because of its low toxicity, biodegradation, biocompatibility, biological activity and stability in blood and cell [3,9,10].

Burn disturbs the balance between the formation of reactive oxygen species and the capacity of antioxidant system in several ways [11]. At high levels of oxidative stress, the significant imbalance between free radicals and antioxidant defense system can lead to cell injury. Recent research has drawn attention to antioxidant peptides [12]. Antioxidant peptides, such as the hydrolysate of hordein [12], soybean [13], collagen [14], are able to suppress the oxidative damage by quenching the free radical chain reactions.

Collagen is the extracellular protein characterized by a structure in triple helices [15]. It has been a widely utilized biomaterial in food, cosmetic, biomedical and pharmaceutical fields [16] due to its favorable biodegradability, biocompatibility, antioxidant [17] and weak immunogenic reaction [18]. Many collagen products for wound healing have been developed in the past few years. Some of them have been approved and are now commercially available [3]. Collagen peptide (COP) as a functional ingredient [19] is hydrolyzed from collagen. Compared with collagen, COP is low molecular weight, which can be easily absorbed by human [20]. It is beneficial to bone, achilles tendon, and skin [20,21]. Additionally, COP has the ability to promote cell attachment and proliferation [22].

To make the best of both biomaterials, researchers have devoted to conjugate chitosan derivatives with variety of peptides. However, the use of traditional crosslinking agents may lead to toxic side effects (owing to residual crosslinking agents) or to secondary reaction with unwanted products [23]. To overcome

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http://dx.doi.org/10.1016/j.reactfunctpolym.2014.01.006
1381-5148/

these problems, recent researches have focused on enzyme-catalyzed reactions which show a variety of benefits, including the high efficiency, selectivity, environmental friendliness [24]. Transglutaminase catalyses an acyl transfer reaction in which the γ-carboxyamide groups of peptide-bound glutamine residues act as acyl donors [25,26], and the primary amino groups of some naturally occurring polyamine, such as chitosan and its derivative [27–29] act as acyl acceptor. Sang et al. [30] synthesized the chitosan–collagen antimicrobial copolymer with microbial transglutaminase (MTGase) as catalyst, and it had been used in protein crosslinking in the food industry. And also Song et al. [31] confirmed that MTGase improved the flavour characteristics and did not affect the antioxidant activity of Maillard Reaction Products. However until now, the preparation of chitosan derivative grafting COP with MTGase as catalyst has never been reported.

In this research, COP–CMC was prepared by catalyzing CMC and COP with MTGase, and the reaction conditions were optimized. Then the H_2O_2 scavenging activity of COP–CMC was investigated. In addition, methyl thiazolyl tetrazolium (MTT) assay evaluated the impact of COP–CMC on proliferation of L929 mouse fibroblasts.

2. Experiment

2.1. Materials

Chitosan (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. Monochloro 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. Preparation of CMC

CMC were prepared according to the previous research with slight modification [32]. Briefly, chitosan (10 g) was added into 50% sodium hydroxide (NaOH) solution prepared previously and was frozen for 24 h. Then monochloro acetic acid (15 g) was added into the thawed chitosan dispersed in isopropanol. And the mixture was stirred vigorously. The carboxymethylation reaction was kept at room temperature for 5 h. The reaction product was dialyzed against distilled water for 3 d through the 8000–10,000 molecular weight cut-off dialysis tubing, and freeze-dried to obtain the purified water-soluble CMC. And the degree of substitution (DS) of CMC was determined according to the acid–base titration method.

2.3. Purification of MTGase

MTGase was first poured into 0.2 mol/L Na_2HPO_4/NaH_2PO_4 buffer solution (PBS, pH 6.0). 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. The specific activity of the lyophilized powder was 53.68 U/mg protein.

2.4. Synthesis of COP–CMC

In a typical reaction procedure, the prepared CMC (1 g) and COP (1 g) were dissolved in PBS (0.2 mol/L, pH 6.0) to prepare aqueous solution respectively. Then they were mixed together to form homogeneous solution. Thereafter MTGase powder (0.1 g) was also dissolved in PBS solution and added to the above mixture to catalyze the reaction between CMC and COP. Magnetic stirring was continuous for 6 h at 50 °C. Then the solution was treated in boiling water for 10 min and later cooled to room temperature. The COP–CMC solution was obtained after filtering with cup-type ultrafilter. 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 dialyses tubing for three days. The dialyzed product was finally freeze-dried to obtain the purified COP–CMC. The dried products were stored in vacuum desiccators over P_2O_5 for further analysis. Scheme 1 illustrates the CMC modified with COP by using MTGase as bio-catalyst.

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

Four independent variables were investigated, including the reaction time (1, 2, 4, 6 and 8 h), the reaction temperature (20, 30, 40, 50 and 60 °C), the molar ratio of CMC to COP (0.0009, 0.0012, 0.0015, 0.0018 and 0.0021) and the pH value (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0). Their variables were fixed at a certain value, while changing only one variable value [13].

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

FT-IR spectra of COP–CMC samples and CMC 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.

2.7. Measurement of degree of substitution

The degree of substitution (DS) is defined as the number of amine groups substituted per repeating structural unit of CMC. In this work, the DS was measured according to the method of Fan [20], the concentration of COP between 0.001 mg/ml and 0.05 mg/ml was liner relation with absorbance at 200 nm by ultraviolet spectrophotometry. And the standard curve for the liner relation was described as Eq. (1). The CMC and COP–CMC samples were also measured with absorbance at 200 nm by ultraviolet spectrophotometry with the concentration of 0.05 mg/ml. The CMC sample was the blank control. The DSs of COP–CMC were determined by Eq. (2).

$$A = 25.322\ C + 0.0097 \quad (1)$$

$$R^2 = 0.9996$$

$$\mathrm{DS} = 333\ C/(40 - 799\ C) \quad (2)$$

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

2.8. Preparation of COP–CMC with different molecular weights

COP–CMC was degraded by an oxidative method involving H_2O_2 [33]. The degradation was carried out as follows: the COP–CMC (1 g) was dissolved in 45 ml distilled water. Afterwards, H_2O_2 (30%, W/W) of a desired volume was added to the above solution and the mixture was stirred at 50 °C for 4 h. Then the reaction was maintained for 20 min at 80 °C to remove the residual H_2O_2. The product was lyophilized. Experiments were conducted under different volume of H_2O_2 to obtain different molecular weights (Mw) of COP–CMC.

2.9. Light scattering measurement [20]

The weight-average molecular weight of COP–CMC was determined with static light scattering. The light-scattering intensities