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Preparation of a bio-composite of sericin-g-PMMA via HRP-mediated graft copolymerization

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ABSTRACT

Silk sericin (SS) derived from silkworms has the characteristics of anti-oxidation, antibacterial, and biocompatibility, however, high solubility in water restricted its applications in biomedical fields. In the present work, SS was enzymatically graft-copolymerized with a hydrophobic vinyl monomer of methyl methacrylate (MMA), through a free radical reaction, through the combination use of hydrogen peroxide and horseradish peroxidase (HRP). Efficacy of the HRP-mediated reaction was examined by means of FTIR, SDS-PAGE, and SEC chromatogram. A bio-composite of SS-*graft*-polymethyl methacrylate (SS-g-PMMA) was constructed subsequently, the corresponding wettability, thermal behavior, and biocompatibility of the obtained composite were examined, respectively. The data reveal that MMA was successfully copolymerized with the reactive sites in sericin chains, resulting in a noticeable increase in the molecular weight. For the membrane of SS-g-PMMA, the surface hydrophobicity was evidently improved compared to that of the untreated, according to the determined data of water contact angle and dissolution ratio. The current work develops an eco-friendly technique for reuse of the industrial waste like sericin, and provides a novel method for preparation of the sericin-based biomaterials as well.

1. Introduction

Silk Sericin (SS), derived from silkworm cocoons is a highly hydrophilic macromolecular protein. It accounts for approximately 20 to 30% of the total silk proteins, wrapping on the surface of silk fiber and protecting the internal component of silk fibroin [1,2]. Sericin proteins contain >18 kinds of amino acids, and some polar side residues from tyrosine, serine, and aspartic acid randomly distribute in the sericin chains [3,4]. While degumming with alkali or protease, the globular proteins of SS are separated from silk fibers, for improving the luster, softness, and dyeability of silk fibroin. The water-soluble sericin proteins, with low crystallinity are always discarded directly into wastewater as a by-product of textile industry, and up to 50,000 t of sericin are produced each year worldwide. Recently, many researchers reported that SS had many important biological activities, such as resisting oxidation, UV-resistant, and antibacterial ability [5–7], and it can be recycled and reused as a naturally finishing agents. Moreover, SS exhibits satisfactory cytocompatibility and low immunogenicity [8,9], and has the potential to be regenerated into diverse materials for biomedical and clinical applications, involving wound healing, drug delivery, skin tissue repair and others [10–12].

Performances of the sericin-based biomaterials highly depend on its morphological and structural characteristics. Unfortunately, the regenerated materials from natural sericin always exhibit poor formability, owing to its amorphous structure and polar amino acid-rich sequences. To overcome the disadvantage of high solubility, many methods were attempted to modify sericin proteins, such as blending with synthetic polymer [13,14], building cross-links using chemicals like gluteraldehyde, genipin, and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) [15–17]. To improve the stability of sericin in water, graft copolymerizations of hydrophobic vinyl monomers onto SS were also carried out by Song et al. [18,19], using the chemical initiators of ceric ammonium nitrate (CAN) and tert-butyl hydroperoxide (TBHP). Chemical modifications can significantly improve the formability of SS, however, some methods may cause considerable inconveniences because of the introduction of chemicals into biomaterials, which will certainly have a negative impact on the biocompatibility of SS and even cause unacceptable damages. Comparatively, enzymatic modifications of protein macromolecules always proceed under mild conditions, offering a safer and eco-friendly alternative for construction of diverse biomaterials.

Horseradish peroxidase (HRP) is one of the most extensively studied peroxidases, it might maintain catalytic activity over broad pH and temperature ranges [20]. In the presence of hydrogen peroxide (H_2O_2) and

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acetyl acetone (ACAC), HRP exhibits the ability of catalyzing the oxidation of phenolic hydroxyl groups in phenols [21]. The possible enzymatic initiation mechanism was proposed by Carlsson et al. [22], HRP can catalyze the main overall reaction: $H_2O_2 + 2AH \rightarrow 2H_2O + 2A^*$, where AH, A* represent the reducing substrate and free radical products, respectively. These free radicals might subsequently initiate the polymerization of phenols or copolymerization with vinyl monomers. In our previous work, HRP-catalyzed graft polymerization of crylic acid (AA) onto silk fibroins was carried out, and efficient apatite deposition onto the composite of SF-g-PAA was observed during the succeeding biomimetic mineralization [23]. Since the content of tyrosine in SS reaches to approximately 5% [24], the phenolic hydroxyl groups in tyrosine residues might be oxidized using the catalytic system of HRP-H_2O_2, and further graft-copolymerized with the vinyl monomer like methyl methacrylate (MMA). This will develop a meaningful way to improve the stability of the sericin-based materials in water.

In the present work, we provide a novel eco-friendly method for preparation of the sericin-based biomaterials. Briefly, enzymatic graft-copolymerization of MMA onto SS was carried out using HRP-H_2O_2 catalytic system, according to the proposed reaction pathway illustrated in Fig. 1. The properties of the obtained SS-g-PMMA composite were concerned, including formability, wettability, thermal stability, and biocompatibility. To our knowledge, little relative study on the enzymatic construction of a bio-composite of SS-g-PMMA was studied before.

2. Materials and methods

2.1. Materials and reagents

Raw *Bombyx mori* silk fibers were purchased from Xinyuan Silk Co. Ltd. (Nantong, P. R. China). HRP (EC1.11.1.7, RZ > 3.0, 300 μ/mg) was available from Aladdin Reagent Co. Ltd. (Shanghai, P. R. China). Methyl methacrylate (MMA) and hydrogen peroxide (30 wt%) were provided by Sinopharm Chemical Reagent Co. Ltd. (Shanghai, P. R. China). P-Hydroxyphenylacetamide (PHAD) as the model compound of tyrosine was purchased from Sigma-Aldrich Corporation. The human osteosarcoma cell line NIH/3T3 was offered from the cell bank of the Chinese Academy of Sciences. Cell counting Kit-8 (CCK-8) was supplied by Beyotime Biotechnology (Shanghai, P. R. China). Acetylacetone (ACAC) and other chemicals were of analytical grade throughout this study.

2.2. Preparation of sericin solution

Degumming of raw silk fibers with 0.1 g/L sodium carbonate was proceeded at 100 °C for 4 h, with a liquor ratio of 1: 50. After removing the degummed silk fibers, the mixture solution was subsequently loaded into dialysis bags (Mw 8000–14,000 kDa), and dialyzed against deionized water to ensure complete removal of salt and small molecular impurities. The concentration of sericin was accurately determined using a weighing method.

2.3. HRP-catalyzed graft copolymerization of SS with MMA

1.0% (w/v) silk sericin was thoroughly mixed with 12 U/mL of HRP, 3 mL/L of ACAC, and 3% (w/v) of MMA in 0.1 mol/L phosphate buffer solution (pH 7.0). Subsequently, 2 mL/L of H_2O_2 (30%) was added dropwise within 1 h, and the enzymatic reaction was initiated in a three-necked flask at 37 °C for up to 4 h. The reaction was proceeded under nitrogen atmosphere to prevent the free radicals from being oxidized. For comparison, individual incubation of SS with HRP-H_2O_2 system was also performed under the same conditions, and the obtained products was referred to as SS-SS. Besides, enzymatic treatment of 2.5 g/L PHAD and 3% w/v MMA were also carried out separately or together under the above-mentioned conditions.

2.4. Preparation of sericin and SS-g-PMMA membranes

After HRP-catalyzed graft copolymerization, the reaction mixture solution was stored at −20 °C for 4 h, then freeze-dried at −50 °C for 24 h. The freeze-dried membrane was immersed in 75% alcohol for 30 min [25], followed by extraction with acetone for 48 h to ensure complete removal of the homopolymer of PMMA. Meanwhile, SS membrane as a control was also prepared according to the above described method. Grafting ratio of MMA onto SS was measured by a weighing method and calculated according to Eq. (1).

$$\text{Grafting ratio } (\%) = \frac{M_2 - M_1}{M_1} \times 100 \quad (1)$$

where M_1 is the total quantity of sericin in the reaction bath, and M_2 is the absolute dry weight of the copolymer of SS-g-PMMA after extracted by acetone.

2.5. ^{1}H nuclear magnetic resonance (^{1}H NMR)

40 mg insoluble copolymerization products of PHAD and MMA were dissolved in 450–500 μL of deuterated dimethyl sulfoxide d_6 (DMSO d_6). Based on the internal standard of tetramethylsilane (TMS), the ^{1}H NMR spectra of each sample was examined in 400 MHz on a Bruker Avance III spectrometer (Bruker, Germany) [26].

2.6. Matrix-assisted laser-desorption/ionization time-of-flight mass spectra (MALDI-TOF MS)

The structures of the diverse compounds produced from the copolymerization of PHAD and MMA were examined, using a MALDI-TOF/TOF instrument (Bruker Daltonics). Each copolymer sample was dissolved

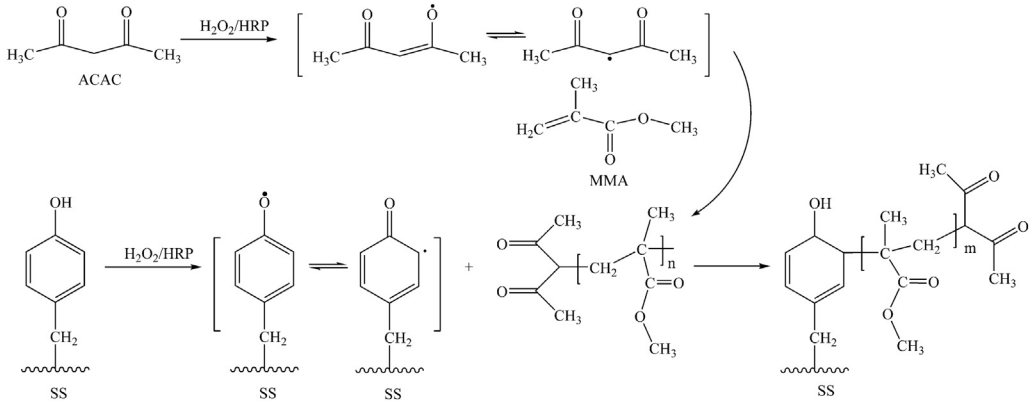

Fig. 1. Proposed mechanism of HRP-catalyzed graft copolymerization of methyl methacrylate (MMA) onto silk sericin.