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Stabilization of recombinant spider silk in thermo-oxidative degradation: High-throughput screening for antioxidants

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ABSTRACT

Recombinant spider silk has arisen as one of the most promising nature-derived building blocks owing to its extraordinary mechanical properties, while its instability against thermo-oxidative degradation becomes a major drawback toward industrial applications. Here, we have firstly implemented a high-throughput screening of antioxidants on the stabilization of recombinant spider silk at an elevated temperature. The usage of high-throughput chemiluminescence imaging has allowed us to screen antioxidants with a wide spectrum of molecular structures and quickly provided two good candidates to stabilize recombinant spider silk powder through an impregnation process: E310 and BHT. An accelerated aging has further proven that these antioxidants suppressed the thermo-oxidation of recombinant spider silk through scavenging the formed radical species and slowed down the formation of carbonyl groups as oxidation products. In addition, we have employed a solution mixing process to further improve the stabilization efficacy of the antioxidants and this method also extended our selection on effective antioxidants, including vitamin E, AO-30, AO-40, HP-10, and especially Irganox 1098. These screening results provide guidelines of selecting or even developing potential antioxidants for stabilizing protein materials.

1. Introduction

The research field of degradation and stabilization is one of the most important aspects in materials science, without which no new materials can be served in our daily life [1–3]. One ultimate goal in this field is to validate the lifetime of a material at the shortest duration: A material is exposed to an environment that mimics the actual service until the "deterioration" is detected [4–9]. An accelerated aging under a more intense environment is a common way to speed up the lifetime evaluation, but the factor of the acceleration is restricted for its accuracy [10,11]. For instance, lifetime prediction often utilizes Arrhenius extrapolation based on accelerated aging data obtained at elevated temperatures, while a discontinuity in the Arrhenius behavior over a wide range of temperatures results in great discrepancy between the estimation and the reality [12]. Thus, the poor throughput of the lifetime evaluation is a definite bottleneck of this field.

Most polymers undergo deterioration in physical properties during processing and services. The mechanism of such deterioration depends on the type of a polymer and an environment that the polymer is subjected to, but mostly falls into oxidation, hydrolysis, thermolysis, proteolysis, photolysis, and their combination [13–18]. The prescription of appropriate stabilizers is the most widely employed strategy to diminish the degradation of polymers, in which the choice of stabilizers primarily depends on the degradation mechanism [19–21]. Commonly, the efficacy of stabilizers cannot be generalized but can only be examined through aging tests for a specific material under a specific aging condition, as it can be affected by many factors, *e.g.* the solubility, migrability, and retentionability in a polymeric host [22–24]. This demanding nature of stabilizer exploration also necessitates a high-throughput methodology. Recently, we have developed a high-throughput chemiluminescence imaging (HTP-CLI) instrument, which enables simultaneous determination of polymer lifetime for 100 samples in a single measurement. The instrument combines an extreme sensitivity of the chemiluminescence (CL) method to detect the

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oxidation of polymers (*ca.* 20 times faster detection was reported in comparison to traditional mechanical tests) [25], and the feasibility of *in-situ* imaging for parallelization. The first demonstration on the stabilization of polypropylene stressed the significance of HTP-CLI in acquiring the lifetime data of 3 years within 1 month [11]. With the given throughput, HTP-CLI is expected to play a vital role in degradation and stabilization research on not only known but also newly developed materials.

Spider silk has been well known to be the strongest among all natural fibers and even rivalling most synthetic fibers [26–29], due to the unique combination of its strength and ductility in a single thread. Together with the excellent mechanical characteristics, the fact that spider silk is composed of ubiquitous light elements (C, O, N, S, etc.) boosts it to be a new class of robust structural materials which are strong, lightweight, and eco-friendly. Spider silk protein can be synthesized through several routes, for examples, genetic engineering in *E. coli* [29–33], transformation of silkworms with chimeric silkworm/spider silk genes [34], production in the milk of transgenic goats [35,36], etc. These progresses nowadays have made it possible to produce recombinant spider silk that equips similar primary structure and somewhat acceptable mechanical strength at a sufficient scale for industrialization [37–39]. Being strongly motivated from those outcomes, scientists and researchers have eagerly brought recombinant spider silk protein into various processes, modification and optimization for advanced applications, such as bullet-proof clothing, wear-resistant lightweight clothing, lightweight vehicle parts, etc. At the same time, the inherent instability of protein against high temperature and humidity [40] results in a noticeable barrier to truly apply recombinant spider silk in practice.

So far, the degradation and stabilization studies on silk materials have been mainly focused on silkworm silk due to its natural abundance and common usages in textiles and medicals. In the textile applications, the interest lies on stabilizers which can prevent photo-degradation and discoloration of silkworm silk [41–43]. Whilst, the medical applications focus more on stabilizers which can control the biodegradability of silk and promote the proliferation of cells in tissue [44]. Most recently, the Kaplan group studied the preservation of natural antioxidants including curcumin and vitamin C inside a silkworm silk film, and reported a positive consequence of strong interaction between the antioxidants and the hydrophobic residues of silk via their aromatic moieties [45]. In our research, we have firstly reported the durability and degradation mechanism of recombinant spider silk situated under a harsh condition, aiming at its applications as industrial building block materials. The studies present that the auto-oxidation mechanism dominates the degradation of recombinant spider silk at an elevated temperature, in which the radical formation initiates at the Cα position of amino acid residues and its unimolecular decomposition affords conjugated carbonyl groups [46,47]. Hence, our next target is to explore stabilizers that can effectively inhibit the thermo-oxidative degradation of recombinant spider silk.

In this study, we have implemented the first extensive exploration on the stabilization of recombinant spider silk against the thermo-oxidative degradation based on HTP-CLI. Effective antioxidants were identified as a result of the implementation at a scale of 10^3 degradation tests by HTP-CLI and subsequent validation using other techniques. A variety of antioxidants including nature-derived antioxidants as well as synthetic hindered phenols, thioethers and phosphites were screened. A key issue was found at the compatibility of antioxidants with employed processes and effective interaction with recombinant spider silk. Potential antioxidants were identified for powder impregnation and solution mixing processes.

2. Materials and methods

2.1. Materials

Recombinant spider silk was synthesized using a similar process to that of Sugahara et al. [48] and provided in an unprocessed powder form by Spiber Inc. The amino acid sequence of the recombinant spider silk mimicked that of *Araneus diadematus*, consisting of Alanine (19.5%), Asparagine (0.5%), Glutamine (17.1%), Glycine (30.9%), Histidine (1.0%), Methionine (0.2%), Proline (14.1%), Serine (9.7%), and Tyrosine (7.0%) at amino acid purity of 95–100%. The average particle size measured by light scattering (Partica LA-950V2, Horiba) was 15.7 μm in the median diameter. The powder was stored in a refrigerator at 4 °C.

Calcium chloride ($CaCl_2$, ≥95%) was purchased from Wako Pure Chemical Industries, Ltd. Common solvents including ethanol (≥99.5%), methanol (≥99.8%) and acetone (≥99.5%) were purchased from Kanto Chemical Co., Inc. Polystyrene (PS) petri dishes (nonsterilized, 35 mmϕ) were purchased from Corning Inc. Dry air (78% of N_2, 21% of O_2 and 1% of Ar) was thoroughly employed in the degradation tests. The antioxidants used in this study are commercially available grades and their structures are shown in Scheme 1a–c. Note that the antioxidants are termed by trivial or trade names for brevity, while their IUPAC names as well as other information is shown in Table S1. Polyamide 6 (nylon 6) pellets were purchased from Sigma-Aldrich Co. An additive-free powder of isotactic polypropylene was obtained ($M_n = 4.6 \times 10^4$, *mmmm* = 98%) by propylene polymerization using a $MgCl_2$-supported Ziegler-Natta catalyst.

2.2. Sample preparation

2.2.1. Stabilization based on powder impregnation

Stabilization of recombinant spider silk powder was carried out by impregnating the powder in a solvent containing a specified amount of an antioxidant. A mixture of acetone and methanol (1/1 v/v) was chosen as a typical example to dissolve an antioxidant at the concentration of 0.1–0.5 mg/mL 2.0 mL of the antioxidant solution was then added to 200 mg of recombinant spider silk powder. The mixture was kept for 16 h under mild stirring and then naturally dried, affording the stabilized powder containing 0.1–0.3 wt % of an antioxidant. All samples were prepared at room temperature and 20–30% R.H. The obtained powder was filled into the wells of a multi-cell plate and subjected to the HTP-CLI measurements.

2.2.2. Stabilization based on solution mixing

A stabilization process based on solution mixing was described as follows. Typically, a dope solution was prepared by dissolving recombinant spider silk powder in a ternary solvent system of $CaCl_2/C_2H_5OH/H_2O$ solution (molar ratio of 1.4:8.0:32.0), yielding a 10.0 wt % solution. An antioxidant that was dissolved separately into ethanol was added into the dope solution to meet the final concentration of 0.2 wt % to spider silk. After mild stirring for 6 h at 65 °C, the dope solution was cast on PS dishes for targeted film thickness of 120 μm. The solution was dried at 550 hPa and 60 °C for 5 days in a vacuum oven system which can process 32 films at once. The obtained gel was immersed in a mixture of acetone and methanol (7/3 v/v) for 30 min and followed by immersing in methanol for another 30 min. The immersing step could cause minor loss of an antioxidant, if it is soluble in methanol or acetone. It is expected that the loss can reduce the actual efficacy of the antioxidant but should not eliminate the stabilization effect, if present. The stabilized spider silk film was naturally dried and then stored in a refrigerator at 4 °C.