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Improved strength of silk fibers in *Bombyx mori* trimolters induced by an anti-juvenile hormone compound

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ABSTRACT

Background: *Bombyx mori* silk fibers with thin diameters have advantages of lightness and crease-resistance. Many studies have used anti-juvenile hormones to induce trimolters in order to generate thin silk; however, there has been comparatively little analysis of the morphology, structure and mechanical properties of trimolter silk.
Methods: This study induced two kinds of trimolters by appling topically anti-juvenile hormones and obtained thin diameter silk. Scanning electron microscope (SEM), FTIR analysis, tensile mechanical testing, chitin staining were used to reveal that the morphology, conformation and mechanical property of the trimolter silk.
Results: Cocoon of trimolters were highly densely packed by thinner fibers and thus had small apertures. We found that the conformation of trimolter silk fibroin changed and formed more β-sheet structures. In addition, analysis of mechanical parameters yielded a higher Young's modulus and strength in trimolter silk than in the control. By chitin staining of silk gland, we postulated that the mechanical properties of trimolters' silk was enhanced greatly during to the structural changes of silk gland.
Conclusion: We induced trimolters by anti-juvenile hormones and the resulting cocoons were more closely packed and had smaller silk fiber diameters. We found that the conformation of trimolters silk fibroin had a higher content of β-sheet structures and better mechanical properties.
General significance: Our study revealed the structures and mechanical properties of trimolter silk, and provided a valuable reference to improve silk quality by influencing molting in silkworms.

1. Introduction

Silkworm silk has a long history of application in the textile industry and is one of the most extensively investigated types of natural silk. In recent decades, silkworm silk has been exploited for many new applications in non-textile fields, including composite materials, regenerative medicine, military products, and the beauty industry due to its combination of high specific strength, high rupture elongation, environmental stability, and biocompatibility properties [1–3]. However, when compared with another natural fiber, the spider dragline silk, silkworm silk is generally considered to be much weaker and less extensible although its yields is far more than spider dragline silk. Recently, many researchers have been made to enhance the mechanical properties of silkworm silk to approach or exceed that of spider dragline silk [4–11]. Shao et al. found that the mechanical properties of silkworm silk are largely dependent on spinning conditions [4]. For example, silk fibers produced by forced reeling at an appropriate rate showed better mechanical properties than those spun naturally. Some researches revealed that investigated the physical properties of the silk spun by different silkworm species and found that the primary structures of fibroin molecules influenced their material properties [5]. Moreover, some researchers tried to incorporate functional material into silk. For instance, Cheng et al. found an interaction between graphene and fibroin peptides obtained from different silk fibroin domains and considered that the graphene substrate contributed to the increase in strength and resilience of different silk fibroin domains [6].

Abbreviations: AJH, anti-juvenile hormone; FTIR, Fourier-transform infrared spectroscopy; ASG, anterior silk gland; SEM, scanning electron microscope; WAG, wheat germ agglutinin-FITC labeled; III-C, larvae were treated with acetone on day 1 of the 3rd instar stage; III-T, larvae were treated with KK-42 on day 1 of the 3rd instar stage; IV-C, larvae were treated with acetone on day 1 of the 4th instar stage; IV-T, larvae were treated with KK-42 on day 1 of the 4th instar stage; DMA, dynamic mechanical analyzer.

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Furthermore, there are also some reports about regenerated silk fibers. They obtained artificial spider silk and silkworm silk with good biocompatibility and enhanced mechanical properties [7,8]. Other researchers fed silkworm with mulberry leaves or artificial diet pretreated with carbon nanotubes, grapheme or titanium dioxide nanoparticles and found that the insects produced "super silk" fibers with superior mechanical properties, thermal stability and ultraviolet resistant properties [9,10]. Genetic manipulation has been used for a long time to reinventing silk. Over-expression of ion-transporting proteins in the spinning duct of silkworms was found to result in production of stronger silk fibers [11].

Interestingly, some studies have reported that the mechanical properties of silk fibers are closely related to their diameter [12,13]. For example, thin silk fiber show a higher breaking strength than the coarse fiber [12]. This result appears to be related to the fact that thin fibers with a small filament size possess high strength because of the high hydrogen bond energy density [13]. In sericulture, people usually get thin silk fibers by breeding trimolter strains or inducing trimolter by anti-juvenile hormone (AJH) [14–18]. Niu et al. induced the general tetramolter silkworms to be trimolter silkworms by AJH and gained thin filament silk with good reelability, excellent neatness, little filament deviation and good cohesion [16]. They reviewed the induction method of trimolter silkworm, especially highlighted its silk quality and filament [17]. However, some researchers found that an AJH named KK-42 not only contributed to the thin filament silk but probably improved the properties of silk [16,18]. Up to now, many researches has tried to use AJHs to induce trimolter for thin silk. Previous studies were mainly concerned about traditional economic traits of trimolters, there are few studies on the mechanical properties of trimolter silk. Niu et al. obtained trimolter by Jinlu inducer and measured the breaking strength and elongation rate of silk [16], and found the strength of silk was not increased and the elongation rate was reduced. Kataoka et al. induced trimolter by bioactive substances [19], and found the strength of the raw silk was 30% higher in comparison to the control silk. However, these investigations on the mechanical properties of trimolter silk were incomplete. Moreover, the structure of trimolter silk is still unkown.

In this research, we applied KK-42 to induce two kinds of trimolters on the first day of the third instar and day 1 of the fourth instar, respectively. SEM showed the morphological differences of cocoon and silk between trimolters and tetramolters. We counted the duration of instars and economic characters of trimolters and measured the diameter and mechanical properties of the trimolters' silk. Our results suggested that the thin filament silk of trimolter had an enormously improvement in strength and Young's modulus. We also collected the silk glands of larvae to conduct the chitin staining and analyzed the reason why the mechanical properties were enhanced greatly.

2. Materials and methods

2.1. AJH induction of silkworm trimolter

The imidazole KK-42 (1-benzyl-5[(E)-2,6-dimethyl-1,5-hepta dienyl]) (SIMR Biotech Company Limited, Shanghai, China) was dissolved in acetone to a concentration of 1 mg/mL. This solution was used as the AJH treatment of *B. mori* larvae and was applied topically (1 μg per larva) on day 1 of the 3rd or 4th instar stages, as described previously [20]. All larvae were reared on fresh mulberry leaves at 25 °C under a 12-h light/12-h dark photoperiod. In the 3rd instar larvae treatment group (III-T), the silkworms were fed normally from the 1st to 2nd instar. Ninety larvae of uniform development were treated with KK-42 on day 1 of the 3rd instar stage. For the 4th instar treatment group (IV-T), the larvae were fed normally from the 1st to 3rd instar. Ninety larvae were treated as above on day 1 of the 4th instar stage. For controls, 30 larvae were treated with acetone on day 1 of the 3rd instar (III-C) and 30 larvae were treated on day 1 of the 4th instar (IV-C). The development of the treated and control larvae was monitored and the entire larval stage was recorded.

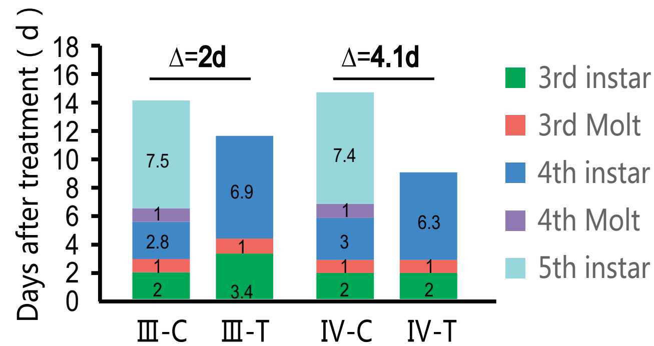

Fig. 1. Schematic diagram of the developmental progression difference after applied KK-42 and acetone separately. III-T and IV-T represents the developmental progression of silkworm applied KK-42 on the day-1 of 3rd and 4th instars respectively, and III-C and IV-C represents that of the silkworm applied the same volume of acetone on the day-1 of 3rd and 4th instars respectively.

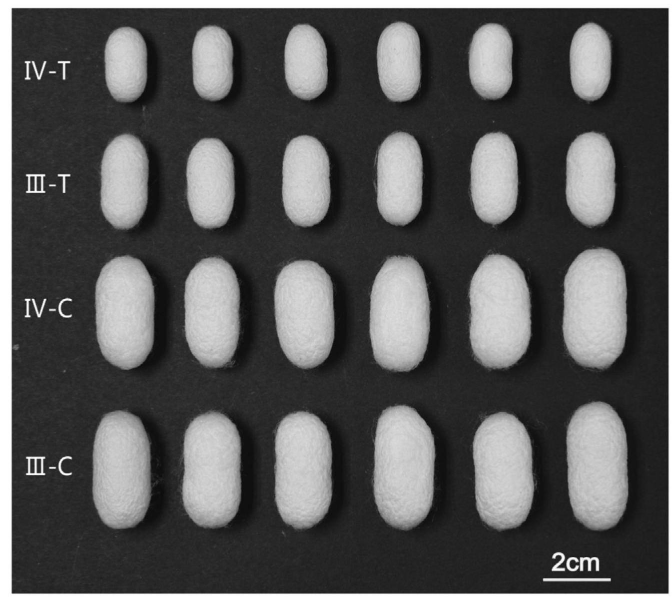

Fig. 2. Photos of cocoons. III-T and IV-T represents cocoons from the silkworm applied KK-42 on the day-1 of 3rd and 4th instars respectively, and III-C and IV-C represents cocoons from the silkworm applied the same volume of acetone on the day-1 of 3rd and 4th instars respectively.

2.2. Chitin staining of the silk gland

Silk glands were fixed overnight in 2.5% glutaraldehyde, washed 3 × 5 min with 150 mM NaCl, 10 mM Na_2HPO4, 10 mM NaH_2PO4 (pH 7.2), then fixed in 30% sucrose glutaraldehyde for 8 h. The anterior silk gland was embedded in O.C.T. compound (opti-mum cutting temperature compound, Sakura, USA). Sections (8 μm) were cut using a Leica CM1950 microtome (Leica Microsystems, Wetzlar, Germany) and washed 3 × 10 min in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 2 mM KH_2PO4, pH 7.4). The sections were incubated with DAPI dihydrochloride solution (Beyotime), washed 3 × 10 min with PBST (PBS containing 0.1% Tween), incubated with wheat germ agglutinin-FITC labeled (WAG) (Sigma), and then washed 3 × 10 min with PBST. The slides were photographed using an Olympus BX51 microscope.

2.3. Scanning electron microscope (SEM) observation

Silk and cocoon samples were randomly obtained from cocoons of