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Novel fabrication of fluorescent silk utilized in biotechnological and medical applications

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ABSTRACT

Silk fibroin (SF) is a natural polymer widely used and studied for diverse applications in the biomedical field. Recently, genetically modified silks, particularly fluorescent SF fibers, were reported to have been produced from transgenic silkworms. However, they are currently limited to textile manufacturing. To expand the use of transgenic silkworms for biomedical applications, a solution form of fluorescent SF needed to be developed. Here, we describe a novel method of preparing a fluorescent SF solution and demonstrate long-term fluorescent function up to one year after subcutaneous insertion. We also show that fluorescent SF labeled p53 antibodies clearly identify HeLa cells, indicating the applicability of fluorescent SF to cancer detection and bio-imaging. Furthermore, we demonstrate the intraoperative use of fluorescent SF in an animal model to detect a small esophageal perforation (0.5 mm). This study suggests how fluorescent SF biomaterials can be applied in biotechnology and clinical medicine.

1. Introduction

Silk fibroin (SF), a natural fibrous protein produced by *Bombyx mori*, has been used for biomedical and biotechnological applications [1]. For example, applications of silk in tissue engineering, wound dressing [2], enzyme immobilization matrices [3], vascular prostheses and structural implants [4,5] have been reported. Depending on its application, SF can be processed into different forms, including film, gel, membrane, powder and porous sponge. However, processing SF into these various forms relies on preparing a solution form of SF as a precursor. To suit a wide range of applications, SF has been integrated with various materials or chemically modified [6]. For example, coupling reactions, amino acid modifications and grafting reactions were used for the chemical modification of silk fibroin. Genetically modified silks produced from transgenic silkworms have recently been reported [7]. Transgenic silkworms can easily be proliferated and retained once the silkworm strain is established and recently, fluorescent transgenic silkworms developed using various transformation vectors [7–9]. Moreover, the transgene inserted into the silkworm genome permits the acquisition of specific desirable characteristics by modifying the silk protein [10,11].

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As is commonly known, green fluorescent protein (GFP), first identified in the aquatic jellyfish *Aequorea victoria*, has been the subject of continued interest since it was cloned in 1992 [12]. Over the decades, fluorescent proteins have become a favorable biotechnological tool that scientists use to investigate the function of genes of interest by directly visualizing, monitoring and quantifying protein expression in living cells. However, there have not been any reports on the biomedical and biotechnological applications using fluorescent silk fibroin. Here, we developed the first method of preparing fluorescent silk fibroin solution in order to produce various fluorescent SF materials.

2. Materials and methods

Silkworm strains. The *B. mori* bivoltine strain, Kumokjam (Jam140 × Jam125), was obtained from the National Academy of Agricultural Science (Suwon, Korea). The silkworms were grown at 25 °C and fed with mulberry leaves and an artificial diet. DNA-injected eggs were maintained at 25 °C in moist Petri dishes. The hatched larvae were fed on an artificial diet and reared in groups under standard conditions.

Plasmid DNA construction. The transition vector pBac-3xP3-DsRed2-FibH was constructed as follows. The DsRed2 cDNA was used as a marker and amplified by PCR using specific primers with *Nhe*I/*Afl*II sites from pDsRed2-C1 (*Nhe*I-DsRed2-F: 5′-GCTAG-CATGGCCTCCTCCGAGAAC-3′ and DsRed2-*Afl*II-R: 5′-CTTAAGCTA-CAGGAACAGGTGGTGGCG-3'; Clontech, Mountain View, CA, USA). The PCR product was cloned into the pGEM-T-easy vector (Promega, Fitchburg, WI, USA) and named pGEM-DsRed2. The DsRed2 gene was excised from pGEM-DsRed2 with *Nhe*I/*Afl*II and replaced with an EGFP gene from pBac-3 × P3-EGFP to generate pBac-3xP3-DsRed2. From the genomic DNA of *B. mori*, the DNA fragment (GenBank Accession No. AF226688, nt. 61,312–63,870), including the promoter domain (1124 bp), N-terminal region 1 (NTR-1, 142 bp), first intron (871 bp), and N-terminal region 2 (417 bp, NTR-2), was amplified by PCR using specific primers with the *Asc*I/*Not*I sites (pFibHN-F: 5′-AGGCGCGCCGTGCGTGATCAGGAAAAAT-3′ and pFibHN-R: 5′-GCGGCCGCTGCACCGACTGCAGCACTAGTGCTGAA-3′). The resultant DNA fragment was cloned into the pGEM-T Easy Vector System (Promega, Fitchburg, WI, USA) and named pGEMT-pFibH-NTR. The DNA fragment (GenBank Accession No. AF226688, nt. 79,021–79,500) including the C-terminal region (179 bp, CTR) and the poly(A) signal region (301 bp) of the H-chain was amplified by PCR using specific primers with the *Sal*I/*Sbf*I/*Fse*I sites (pFibHC-F: 5′-CCTGCAGGAAGTCGAC AGCGTCAGTTACG-GAGCTGGCAGGGGA-3′ and pFibHC-R: 5′-GGCCGGCCTA-TAGTATTCTTAGTTGAGAAGGCATA-3′). The resultant DNA fragment was cloned into the pGEM-T Easy Vector System (Promega, Fitchburg, WI, USA) and named pGEMT-CTR. The pFibH-NTR fragment was excised from pGEMT-pFibH-NTR with *Apa*I/*Sal*I and subcloned into a pBluescriptII SK(−) vector (Stratagene, La Jolla, CA, USA) that had been digested with *Apa*I/*Sal*I to generate pFibH-NTR-null. Next, a CTR fragment was excised from pGEMT-CTR with *Sal*I/*Sac*I and subcloned into a pFibH-NTR-null that had been digested with *Sal*I/*Sac*I to generate pFibHNC-null. Fluorescent genes (EGFP, mKate2, EYFP) were synthesized and purchased from the BIONEER corporation (Korea). The N- and C-terminals had the *Not*I and *Sbf*I restriction sites, respectively. The fluorescent genes were digested with *Not*I/*Sbf*I and subcloned into a pFibHNC-null that had been digested with *Not*I/*Sbf*I to generate pFibHNC-EGFP, pFibHNC-mKate2, and pFibHNC-EYFP. These vectors were then digested with *Asc*I/*Fse*I and subcloned into separate pBac-3xP3-DsRed2. The resulting vectors pBac-3xP3-DsRed2-FibH-EGFP, pBac-3xP3-DsRed2-FibH-mKate2, and pBac-3xP3-DsRed2-pFibH-EYFP were generated. pBac-3 × P3-EGFP[25] and the helper vector pHA3PIG[7] were provided by Dr. M. Jindra (Academy of Sciences of the Czech Republic, Prague, Czech Republic). Each vector was purified with an EndoFree Plasmid Maxi Kit (QIAGEN Gmbh, Hilden, Germany) and used to generate transgenic silkworms.

Transgenesis and screening of silkworms. For egg preparation, male and female moths were allowed to mate for at least 4 h at 25 °C. The mating moths were stored overnight at 4 °C. The female moths were placed on a plastic sheet and left in dark boxes for 1 h. Laid eggs were immersed in HCl (specific gravity 1.0955, 25 °C) for 30 min at 25 °C, rinsed with distilled water, and dried. The transition vectors (pBac-3xP3-DsRed2-pFibH-EGFP, pBac-3xP3-DsRed2-pFibH-mKate2, and pBac-3xP3-DsRed2-pFibH-EYFP) and the helper vector pHA3PIG were dissolved in 5 mM KCl and 0.5 mM phosphate buffer (pH 7.0) at a concentration of 0.2 μg/μL and mixed at a ratio of 1:1. Approximately 5–10 nL of this mixture were injected using an IM300 microinjector (Narishige Scientific Instrument Lab., Tokyo, Japan) into pre-blastoderm embryos at 2–8 h after oviposition. Injected embryos were allowed to develop at 25 °C in moist chambers. G1 embryos and larvae were screened under a fluorescence stereomicroscope equipped with a red filter (Leica, Wetzlar, Germany).

Preparation of fluorescent silk fibroin solution. The fluorescent cocoons were heated overnight at 60 °C in an aqueous solution of 3% $NaHCO_3$ with alcalase (1.5 ml/L) and then washed with distilled water several times to remove the glue-like sericin proteins. Subsequently, the extracted silk was dissolved in a 9.5 M LiBr solution with 1 mM DTT at 40 °C. Then, this solution was filtered through a miracloth (Calbiochem, San Diego, CA, USA) and dialyzed with distilled water for 2 days to remove the salt. The final concentration of the aqueous silk fibroin solution was 4 wt. %. The SF solutions were stored at 4 °C before use to avoid premature precipitation or freeze-dried to obtain regenerated silk fibroin.

Fluorescence studies. The fluorescence spectrum of each cocoon and solution was captured using the LS-55 (Perkin Elmer, Santa Clara, CA, USA) fluorescence spectrophotometer. The fluorescence was recorded at excitation wavelengths of 488 nm–588 nm and emission wavelengths of 507 nm–633 nm.

Preparation of silk fibroin materials. To obtain SF membranes, the SF solution, prepared as described above, was cast on a polystyrene plate at 0.4 ml/cm, followed by drying overnight. SF sponges were obtained from SF solution in a mold, frozen at −80 °C overnight, and freeze-dried for 2 days to completely remove the solvent.

Cell study. The fibroblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml penicillin and 100 μg/ml streptomycin. The membranes were sterilized by soaking the samples in 70% ethanol for 30 min. The wells were seeded at a density of 10,000 cells/well onto fluorescent silk fibroin membranes. The media was changed two times per week. To examine the cell attachment on the SF membrane, cells (10,000 cells/well) were allowed to grow for 24 h. The SF membranes were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer for 30 min, washed repeatedly with PBS buffer, and stained with DAPI (Vector Lab., Burlingame, CA, USA) and rhodamine phalloidin (Invitrogen Co., San Diego, CA, USA). The membranes were analyzed under a fluorescence microscope (Eclipse 80i, Nikon Co., Japan).

Animals. 8-week- old male nude mice (BALB/c), 8-week- old male mice (ICR) and 8-week- old male rats (SD) were purchased from Dooyeol Biotech (Seoul, Korea) and the animal center of Hallym University in Korea, respectively. This study was approved by the Institutional Review Board of Hallym University, Chuncheon, Korea. All animals were observed in separate cages with free access to food and water. All surgical procedures were performed under general anesthesia using intraperitoneal xylazine (5–10 mg/kg) and intramuscular ketamine (40–80 mg/kg).