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Molecular cloning, gene expression analysis, and recombinant protein expression of novel silk proteins from larvae of a retreat-maker caddisfly, *Stenopsyche marmorata*

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ABSTRACT

Retreat-maker larvae of *Stenopsyche marmorata,* one of the major caddisfly species in Japan, produce silk threads and adhesives to build food capture nets and protective nests in water. Research on these underwater adhesive silk proteins potentially leads to the development of new functional biofiber materials. Recently, we identified four major *S. marmorata* silk proteins (Smsps), Smsp-1, Smsp-2, Smsp-3, and Smsp-4 from silk glands of *S. marmorata* larvae. In this study, we cloned full-length cDNAs of Smsp-2, Smsp-3, and Smsp-4 from the cDNA library of the *S. marmorata* silk glands to reveal the primary sequences of Smsps. Homology search results of the deduced amino acid sequences indicate that Smsp-2 and Smsp-4 are novel proteins. The Smsp-2 sequence [167 amino acids (aa)] has an array of GYD-rich repeat motifs and two $(SX)_4E$ motifs. The Smsp-4 sequence (132 aa) contains a number of GW-rich repeat motifs and three $(SX)_4E$ motifs. The Smsp-3 sequence (248 aa) exhibits high homology with fibroin light chain of other caddisflies. Gene expression analysis of Smsps by real-time PCR suggested that the gene expression of Smsp-1 and Smsp-3 was relatively stable throughout the year, whereas that of Smsp-2 and Smsp-4 varied seasonally. Furthermore, Smsps recombinant protein expression was successfully performed in *Escherichia coli*. The study provides new molecular insights into caddisfly aquatic silk and its potential for future applications.

1. Introduction

Caddisflies (order Trichoptera) are a large group of aquatic insects. *Stenopsyche marmorata* (suborder Annulipalpia, called "retreat-maker") is one of the most common large caddisflies in rivers in Japan, such as the Chikuma (Shinano) River [1]. The larvae feed, mature, and pupate underwater and spin aquatic adhesive silk to build essential structures including food capture nets and protective nests [2,3]. Research on silk proteins from caddisfly larvae could lead to novel biopolymer materials for underwater adhesive and biomedical purposes [4]. Recently, we identified four major *S. marmorata* silk proteins (Smsps) extracted from the silk glands of *S. marmorata* larvae [5]. The high-molecular-mass protein (>~300 kDa) was designated as Smsp-1; the three low-molecular-mass proteins were designated as Smsp-2, Smsp-3, and Smsp-4 (~26–32 kDa, ~21–26 kDa, and ~16–17 kDa, respectively, in SDS–PAGE analysis) [3,5,6]. We also reported the biochemical characterization and amino acid sequences of Smsp-1 that are the major component of the larval net silk/adhesive precursor [3,6]. However, detailed molecular information on Smsp-2, Smsp-3, and Smsp-4 is still unknown. To reveal the molecular characteristics of the aquatic silk proteins, we first report the primary sequences of

Abbreviations: DDBJ, DNA data bank of Japan; H-fibroin, fibroin heavy chain; HRP, horseradish peroxidase; IMAC, immobilized metal ion affinity chromatography; IPTG, isopropyl β-D-1-thiogalactopyranoside; L-fibroin, fibroin light chain; MALDI–TOF, matrix-assisted laser desorption/ionization time-of-flight; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Smsp, *Stenopsyche marmorata* silk protein; TEV, Tobacco Etch Virus; TF, trigger factor.

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Smsp-2, Smsp-3, and Smsp-4 deduced from their full-length cDNA clones, which were isolated from the cDNA library of the *S. marmorata* silk glands. In addition, we performed gene expression analysis and recombinant protein expression of Smsps.

2. Materials and methods

2.1. N-terminal amino acid sequencing of Smsps by Edman degradation

The fifth instar larvae of *S. marmorata* were collected in the middle reaches of the Chikuma River in Nagano Prefecture, Japan. The silk glands were dissected from the larvae; the major silk proteins (P3′ fraction) were extracted from the silk glands as previously reported [5,6]. The proteins were separated by SDS–PAGE and blotted onto a PVDF membrane. The individual bands of Smsp-2, Smsp-3, and Smsp-4 were cut out from the membrane; the N-terminal amino acid sequencing was performed on an automated protein sequencer PPSQ-21 (Shimadzu).

2.2. cDNA cloning of Smsps

The cDNA libraries were constructed from silk glands of *S. marmorata* larvae as previously described [6]. In brief, the silk glands were dissected from the larvae; the total RNA from the silk glands was isolated using the ISOGEN reagent (Nippon Gene). The polyA$^+$ RNAs were purified using the Oligotex-dT30 <Super> mRNA purification kit (Takara Bio). The cDNA libraries were constructed using the cDNA library construction kit with a cloning vector pAP3neo (Takara Bio) or the CloneMiner cDNA library construction kit with a cloning vector pDONR222 (Life Technologies).

The 3′ end cDNA fragments of Smsp-2 and Smsp-4 were amplified from the cDNA library (pAP3neo) by polymerase chain reaction (PCR) using KOD -Plus- Neo DNA polymerase (Toyobo) with the degenerate primer, Smsp-2N-FW or Smsp-4N-FW, and the vector-specific primer, T3promoter(pAP3neo). (Table S1.) Each of the amplified DNA fragments of Smsp-2 and Smsp-4 was cloned into the plasmid vector pBluescript II KS(+) (Agilent Technologies) and digested with *Eco*RV by blunt-end ligation. DNA sequence analysis was performed using Applied Biosystems 3130xl Genetic Analyzer (Life Technologies). The 5′ end cDNA fragments were amplified from the cDNA library by PCR with the specifically designed primer, Smsp-2-RV1 or Smsp-4-RV1, and the vector-specific primer, T7FWlongpAP3neo. The amplified 5′ end cDNA fragments of Smsp-2 and Smsp-4 were cloned into pBluescript II KS(+); DNA sequences were analyzed. The 5′ and 3′ ends of cDNA sequences were assembled into the full-length cDNA sequences.

The cDNA clone of Smsp-3 was isolated from the cDNA library (pDONR222) using PCR with the vector-specific primer M13FW and the designed degenerate primer Smsp-3-RV1.

The determined cDNA sequences were registered in DNA data bank of Japan (DDBJ) with accession numbers, LC057251 for Smsp-2, LC057252 for Smsp-3, and LC057253 for Smsp-4.

2.3. Western blotting to detect phosphoserine

Western blot analysis was performed using rabbit anti-phosphoserine (Life Technologies) primary and F(ab')$_2$-goat anti-rabbit IgG (H + L) HRP-conjugated (Life Technologies) secondary antibodies. Immunoreactions were visualized by Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore).

2.4. Mass spectrometry

After SDS–PAGE of the P3′ fraction from the silk glands (Fig. S1), the gel bands of Smsp-3 and Smsp-4 stained by the reverse staining method [7] were excised, destained, and crushed. The whole proteins were eluted from the gel fragments by formic acid/water/2-propanol (1:3:2 v/v/v) [8] and analyzed using a MALDI-TOF mass spectrometer, TOF/TOF 5800 System (AB SCIEX) using a sinapic acid matrix.

2.5. Gene expression analysis of Smsps by real-time PCR

Fifth instar larvae of *S. marmorata* were collected around Kamuriki Bridge in the middle part of the Chikuma River in Nagano Prefecture, Japan, every month from May to December 2012 (Table S2). The silk glands were dissected from the larvae; total RNA from the silk glands was extracted using a High pure RNA tissue kit (Roche Diagnostics). Quantitative real-time PCR was performed on a MiniOpticon Real-Time PCR System (Bio-Rad). The cDNA template, the forward and reverse primers for each Smsp (Table S3), and SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio) in a total of 25 μl were applied to the following PCR programs: 95 °C for 30 s (initial denaturation); 95 °C for 5 s and 60 °C for 30 s, repeated for 40 cycles (amplification). The ribosomal protein genes *rpL11 and rpL31* from *S. marmorata* (DDBJ accession numbers LC057254 and LC057255, respectively) were used for normalization.

2.6. Recombinant protein expression in Escherichia coli

Each of the cDNA encoding Smsps without the secretory signal sequence was cloned into pENTR-TEVL, a modified pENTR vector with a Tobacco Etch Virus (TEV) protease cleavage site, derived from pENTR1A (Life Technologies). The cDNA of Smsp-1 (clone 3–54) encoded the C-terminal fragment region, Smsp-1c (479 residues) [6]. The cDNA of Smsp-4 was modified with codon optimization for expression in *E. coli*. The protein expression vectors pDEST17-Smsps and pCold-TF-Smsps were constructed using GATEWAY technology (Life Technologies) with pENTR-TEVL-Smsps and pDEST17 (Life Technologies) or pCold-TF-GW, which was the modified pCold TF (Takara Bio) with a GATEWAY reading frame cassette (Life Technologies). The Smsps with a His_6 tag (H-Smsps) were expressed in *E. coli* BL21 Star (DE3) (Life Technologies) harboring pDEST17-Smsps (with a T7 promoter) using LB broth (50 μg/mL ampicillin) at 37 °C for ~7 h. For the Smsps fusion protein with a His_6 and trigger factor (TF) tag (TF-Smsps), *E. coli* BL21 Star (DE3) harboring pCold-TF-Smsps (cold shock promoter) was cultured using LB broth (50 μg/mL ampicillin) at 37 °C for ~2 h. At OD_{660} = ~0.5, expression was induced with 50 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) and cold shock at 15 °C; the cells were further cultured for 16 h at 15 °C. The harvested cells were disrupted by sonication in a lysis/wash buffer (50 mM sodium phosphate buffer (pH 7.0) containing 300 mM NaCl, 10% glycerol). The soluble and insoluble fractions were separated by centrifugation. The proteins including H-Smsps in the insoluble fractions were solubilized with a solubilization/wash buffer (20 mM Tris–HCl buffer (pH 8.0) containing 8 M urea, 1 mM dithiothreitol, and 1 mM EDTA). The proteins were purified by immobilized metal ion affinity chromatography (IMAC) with cOmplete His-tag purification resin (Roche Diagnostics) for H-Smsps or TALON metal affinity resin (Takara Bio) for TF-Smsps; the proteins were eluted using 250 mM imidazole in the lysis/wash buffer or the solubilization/wash buffer.

3. Results and discussion

3.1. cDNA cloning and amino acid sequences of Smsp-2 and Smsp-4

For cDNA cloning of Smsp-2 and Smsp-4, the degenerate primers, Smsp-2N-FW and Smsp-4N-FW (Table S1), were designed based on the N-terminal amino acid sequences of Smsp-2 and Smsp-4 by Edman degradation sequencing (Table S4). The full-length cDNA clones of Smsp-2 and Smsp-4 (Figs. S2 and S3) were successfully isolated from the cDNA library of the *S. marmorata* silk glands by PCR cloning. Homology search results of basic local alignment search tool (BLAST) [9] showed no homologous proteins with significant similarity, indicating that Smsp-2 and Smsp-4 are novel proteins.

Fig. 1A shows the deduced amino acid sequence of Smsp-2. The full-length Smsp-2 protein is composed of 167 amino acid residues including the N-terminal secretory signal sequence (19 residues) predicted by SignalP [10] (Fig. S4A). The subsequent sequence is essentially consistent with the N-terminal amino acid sequence (Fig. 1A and Table S4). The experimental analysis of the amino acid composition indicated that Smsp-2 highly contained Gly (25.4 mol %), Tyr (16.9 mol%), and Asx (9.9 mol%) [3,6], which is consistent with that deduced from the cDNA of Smsp-2 (Table S5). The molecular mass of Smsp-2 without the signal sequence is calculated to be 15.7 kDa, which is lower than ~26–32 kDa estimated by SDS–PAGE, possibly implying potential post-translational modifications and/or unusual mobility shift on electrophoresis. Smsp-2 has an array of unusual GYD-rich repeat motifs comprising Gly, Tyr, and Asp, and two $(SX)_4E$ motifs (Fig. 1A and Fig. S5A). Our recent study on Smsp-1 showed that many Ser residues of $(XS)_n$ motif in Smsp-1 were *O*-phosphorylated, and the *O*-phosphoserine residue occurred in a clustered manner, probably serving a cement function for Smsp-1 [6]. In addition, a repeating $(SX)_n$ motif conserved in the fibroin heavy chain (H-fibroin) of a case-maker larva of caddisfly (suborder Integripalpia), *Hesperophylax consimilis* (corrected in the subsequent paper [11]), was also densely phosphorylated [12]. These data suggest that the Ser residues of the $(SX)_4E$ motifs of Smsp-2 are potential *O*-phosphorylation sites.

Fig. 1B shows the deduced amino acid sequence of Smsp-4. The full-length Smsp-4 protein is composed of 132 amino acid residues including the N-terminal secretory signal sequence (19 residues), which is suggested by the N-terminal amino acid sequence (Table S4) and predicted by SignalP [10] as the second candidate (Fig. S4C). The subsequent sequence is mostly consistent with the N-terminal amino acid sequence (Fig. 1B and Table S4). The experimental analysis of the amino acid composition indicated that Smsp-4 highly contained Gly (20.3 mol%), Trp (8.9 mol%), His (7.5 mol%), and Ser (7.3 mol%) [3,6], which is roughly consistent with that deduced from the cDNA of Smsp-4 (Table S5). The molecular mass of Smsp-4 without the signal sequence is calculated to be 12.4 kDa, which is a roughly reasonable value compared to ~16–17 kDa estimated by SDS–PAGE considering experimental errors and/or potential post-translational modifications. As shown in Fig. 1B and Fig. S5B, Smsp-4 has a significant number of

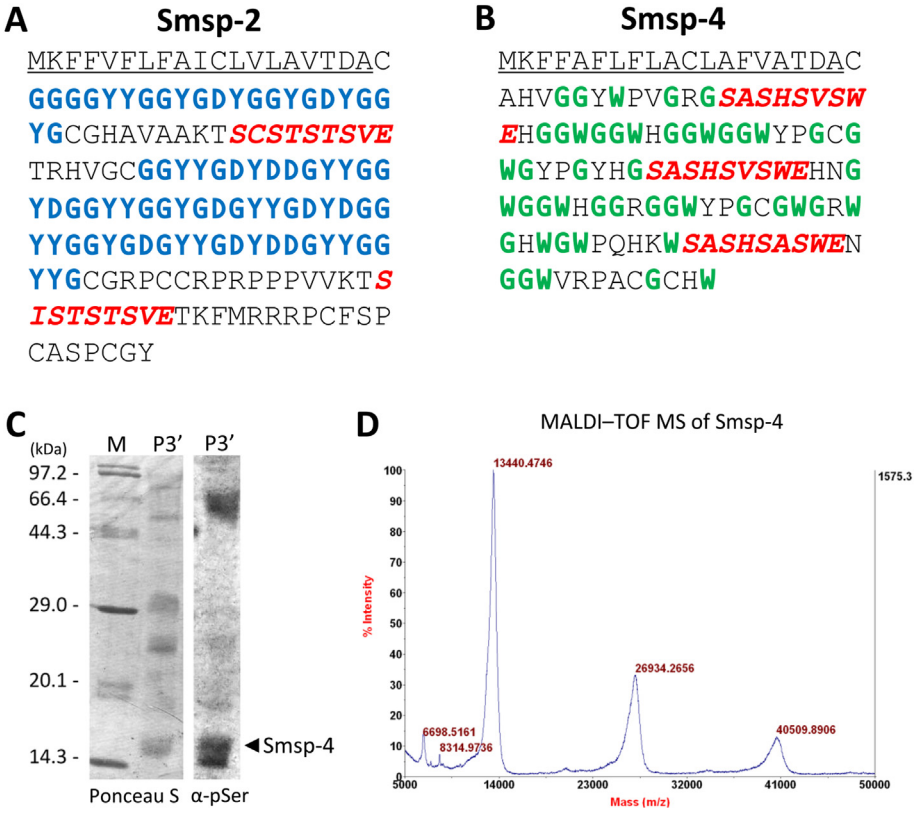

Fig. 1. The novel silk proteins Smsp-2 and Smsp-4 from *S. marmorata*. (A) The deduced amino acid sequence of Smsp-2. The secretory signal sequence predicted by SignalP is underlined. The GYD-rich repeat motif and the $(SX)_4E$ motif are shown in blue bold and red italic fonts, respectively. (B) The deduced amino acid sequence of Smsp-4. The predicted secretory signal sequence is underlined. The GW-rich repeat motif and the $(SX)_4E$ motif are shown in green bold and red italic fonts, respectively. (C) Western blot analysis of Smsp-4. Phosphorylation of Ser was detected with an anti-phosphoserine antibody (right). All proteins on the PVDF membrane were stained with *Ponceau S* (left). M: molecular mass marker; P3′: the P3′ fraction from the *S. marmorata* silk glands [5,6]. (D) The MALDI–TOF mass spectrum of the Smsp-4 protein extracted from the *S. marmorata* silk glands. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

characteristic GW-rich repeat motifs comprising Gly and Trp. Also Smsp-4 has three $(SX)_4E$ motifs, which are suggested as potential *O*-phosphorylation sites. Western blot analysis with an anti-phosphoserine antibody suggested that Smsp-4 was phosphorylated at Ser residues (Fig. 1C). Furthermore, the MALDI–TOF mass spectrum of the Smsp-4 protein extracted from the silk glands (Fig. 1D) shows that the first main peak of *m/z* 13440.5 is probably assignable to a fully phosphorylated Smsp-4 monomer (~13.4 kDa) considering mass increase due to phosphorylation of twelve Ser residues ($+80$ Da $\times$ 12 $= +960$ Da: twelve Ser residues of potential phosphorylation sites at three of the $(SX)_4E$ motif in Smsp-4). The asymmetric shape of the relatively-broad mass peak with a lower-mass tail probably suggests its heterogeneity of partial phosphorylation. Also, the second and third peaks of *m/z* 26934.3 and 40509.9 are assignable to the phosphorylated Smsp-4 dimer and trimer, respectively, suggesting that Smsp-4 potentially forms homo-oligomers possibly because the GW-rich repeats interact with each other by stacking and hydrophobic interactions of Trp residues.

3.2. cDNA cloning and amino acid sequence of Smsp-3

As in the case of Smsp-2 and Smsp-4, we first tried cDNA cloning of Smsp-3 from the cDNA library by PCR with the degenerate primers, Smsp-3N-FW or Smsp-3N-FW2 (Table S1), designed based on the N-terminal amino acid sequence of Smsp-3 (Table S4). However, we attempted unsuccessfully to obtain a cDNA fragment of Smsp-3. Then, we changed to another strategy for the cDNA cloning of Smsp-3. Because the N-terminal amino acid sequence of Smsp-3 shares significant homology with fibroin light chain (L-fibroin) from other caddisflies (Fig. S6A), we tried cDNA cloning of L-fibroin from *S. marmorata* using the sequence alignment information of L-fibroin from other caddisflies. We designed another degenerate primer, Smsp-3-RV1 (Table S1), based on the highly homologous sequence region "NN(V/I)GAAATSAAT" found in the sequence alignment of L-fibroin from three caddisfly species, *Limnephilus decipiens*, *Rhyacophila obliterate*, and *Hydropsyche angustipennis* [13,14] (Fig. S6B). The full-length cDNA clone of *S. marmorata* L-fibroin (Fig. S7) was successfully isolated from the cDNA library of the silk glands by PCR with the new degenerate primer. Finally, we confirmed that Smsp-3 was identical to *S. marmorata* L-fibroin because the tryptic-digested Smsp-3 was significantly identified as *S. marmorata* L-fibroin (the amino acid sequence deduced from the cloned cDNA) by using tandem mass spectrometry (Fig. S8).

Fig. 2 shows the deduced amino acid sequence of Smsp-3 (*S. marmorata* L-fibroin) on the sequence alignment of L-fibroin from other caddisfly species. The full-length Smsp-3 protein is composed of 248 amino acid residues including the predicted N-terminal secretory signal sequence (18 residues) (Fig. S4B). The molecular mass of Smsp-3 without the signal sequence is calculated to be 24.1 kDa, which corresponds to ~21–26 kDa estimated by SDS–PAGE. In addition, the molecular mass of the whole protein of Smsp-3 extracted from the silk gland was measured by MALDI–TOF mass spectrometry (Fig. S9). The doublet peak of *m/z* 23291.8 and 23482.6 is probably assignable to the Smsp-3 monomer considering experimental errors. As shown in Fig. 2, Smsp-3 shares significantly high homology with L-fibroin from other caddisflies, *H. angustipennis* (71% identities), *Hesperophylax occidentalis* (54%), *L. decipiens* (54%), and *R. obliterata* (51%), suggesting that Smsp-3 has the same function as L-fibroin of other caddisflies.

3.3. Gene expression analysis by real-time PCR

Using real-time PCR, we performed gene expression analysis of Smsps in the silk glands from natural larvae samples taken every month from May to December (Table S2) (Fig. 3). The results show that the Smsp-1 and Smsp-3 genes were expressed at relatively

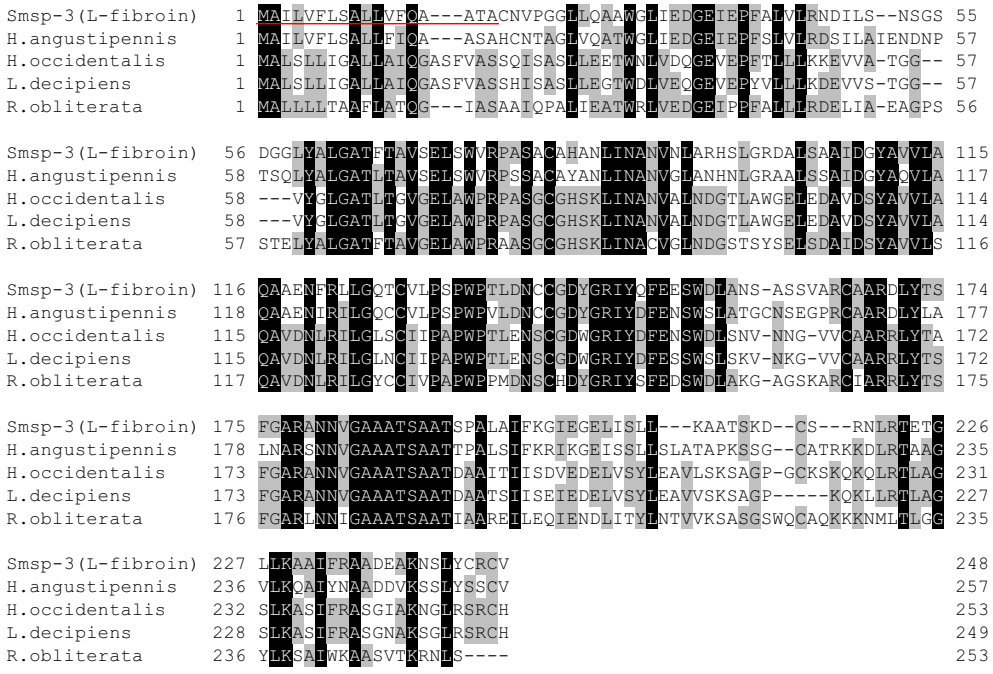

Fig. 2. The deduced amino acid sequence of Smsp-3 (*S. marmorata* L-fibroin) on the sequence alignment of L-fibroin from other caddisfly species. The secretory signal sequence of Smsp-3 predicted by SignalP is underlined in red. The amino acid residues completely conserved are highlighted in black. The amino acid residues conserved with 60–80% similarities are highlighted in gray. Accession numbers for L-fibroin: *Hydropsyche angustipennis*, BAF62094 [13]; *Hesperophylax occidentalis,* AlO11229 [17]; *Limnephilus decipiens,* BAF62096 [13]; *Rhyacophila obliterate,* BAH80180 [14]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

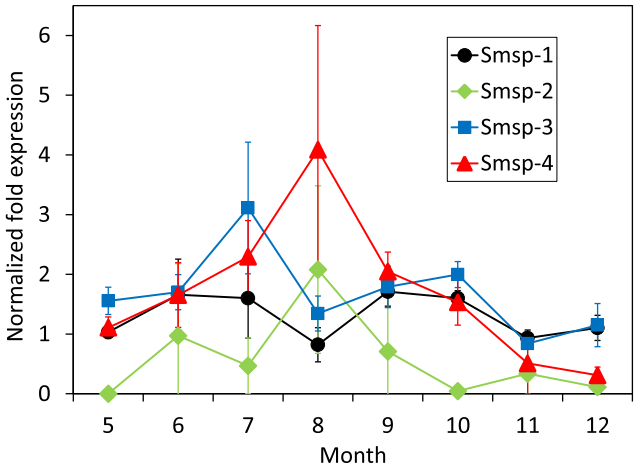

Fig. 3. The gene expression analysis of Smsps from natural samples taken every month from May to December (Table S2). The expression data were normalized to the value of Smsp-1 in May. Error bars represent ±1 standard deviation (n = 2–4).

stable levels, irrespective of seasons. In contrast, the expression levels of the Smsp-2 and Smsp-4 genes were higher in summer and lower in winter (significant difference between July–August and November–December in Smsp-2 ($p < 0.05$) and in Smsp-4 ($p < 0.01$)). However, the expression pattern of Smsp-2 was somewhat elusive, and it was possibly controlled by on/off-like regulation. These results suggest that Smsp-1 and Smsp-3 play fundamental roles as backbones of the silk protein complex through all seasons. However, Smsp-2 and Smsp-4 may play additional roles because the Smsp-4 expression varied seasonally, and the Smsp-2 expression was strictly controlled as may be necessary.

3.4. Recombinant protein expression of Smsps in E. coli

We constructed recombinant protein expression systems of Smsps in *E. coli* to facilitate further research and future applications. First, we tried T7 expression system for Smsps with a His_6 tag (H-Smsps). All H-Smsps were expressed in the insoluble fractions with and without IPTG induction (Fig. S10A). H-Smsp-3 and H-Smsp-4 were highly expressed. The H-Smsps proteins were successfully solubilized and purified by IMAC under denaturing conditions with 8 M urea (Fig. 4A). Second, we tried cold shock expression system for Smsps with a His_6 tag and TF tag (TF-Smsps). TF-Smsp-2, TF-Smsp-3, and TF-Smsp-4 were expressed in soluble fractions with IPTG and cold shock induction (Fig. S10B). TF-Smsp-3 and TF-Smsp-4 were highly expressed in the soluble fractions and successfully purified by IMAC under native conditions (Fig. 4B). However, TF-Smsp-2 was hardly purified and TF-Smsp-1c could not be purified in the soluble fractions, probably due to protein degradation.

3.5. A putative complex model of Smsps with various interactions

The gene expression analysis suggests that Smsp-1 and Smsp-3 play fundamental roles as backbones of the silk protein complex through all seasons. In the domestic silkworm *Bombyx mori*, H-fibroin and L-fibroin form a complex by a disulfide linkage between Cys-c20 of H-fibroin and Cys-172 of L-fibroin [15]. Amino acid sequences of H-fibroin and L-fibroin from *B. mori* around these regions were homologous to H-fibroin and L-fibroin from caddisflies, *H. angustipennis* and *L. decipiens* [13], and also to Smsp-1 and Smsp-3 from *S. marmorata*, respectively (Fig. S11). The two Cys residues of Cys-c20 (twentieth amino acids from C-terminal) of H-fibroin and Cys-172 of L-fibroin from *B. mori* are strictly conserved among these caddisflies including *S. marmorata*, suggesting that Smsp-1 and Smsp-3 also potentially form a heterodimeric complex by a disulfide linkage between the two Cys residues (Fig. S11).

The western blot analysis with anti-phosphoserine antibody (Fig. 1C) and the mass spectrum of Smsp-4 (Fig. 1D) suggest that the Ser residues of Smsp-4 are phosphorylated probably at the $(SX)_4E$ motif, similar to Smsp-1. In our previous study, the addition of EDTA induced the separation of Smsp-1 from the other proteins containing Smsp-2, Smsp-3, and Smsp-4 [6]. These results suggest that the complex formation of Smsp-1 and Smsp-4 is driven by cross-bridging of the anionic phosphoserine clusters, the $(pSX)_n$ motifs of Smsp-1 and Smsp-4 together with the cationic metal ions such as Ca^{2+}. In the silk from a case-maker caddisfly, *H. consimilis*, the structural model, in which the phosphorylated serine repeats $(pSX)_4$ complex with divalent cations Ca^{2+} and Mg^{2+} to form rigid nanocrystalline β-sheet structures, was also reported [16].

The Smsp-2 expression was strictly controlled, suggesting that Smsp-2 is not a major component of the silk but an additional and optional factor. The newly-discovered GYD-rich repeat motif of Smsp-2 implies its unique function, which is probably related to its surprisingly-high composition of Tyr (20.3%) (Table S5). Recently, dityrosine crosslinking, catalyzed by peroxinectin in the adhesive underwater silk of a case-maker caddisfly, *H. occidentalis*, was reported [17]. The Smsp-2 with many Tyr residues at the GYD-rich repeat motif may be a potential substrate protein dityrosine-crosslinked by peroxinectin to enhance the molecular network and mechanical properties of caddisfly silk fibers.

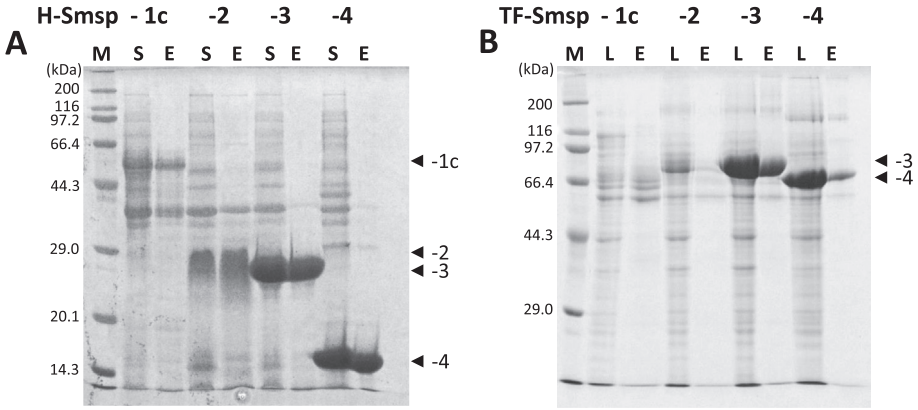

Fig. 4. Recombinant protein expression of Smsps in *E. coli*. (A) SDS–PAGE (15% gel) of His_6-tagged Smsps (H-Smsps). (B) SDS–PAGE (10% gel) of trigger factor-tagged Smsps (TF-Smsps). M: molecular mass marker; S: solubilized samples from the insoluble fractions; E: eluted samples after IMAC purification; L: cell lysate samples. Proteins were stained with Coomassie brilliant blue.

The Smsp-4 expression varied seasonally and tended to be higher in summer and lower in winter, suggesting that Smsp-4 plays more important roles in summer than in winter. Since the caddisfly larvae live an active life in summer, they produce more silk fibers in summer than in winter, implying that Smsp-4 may be associated with efficient silk production. In the silkworm *B. mori*, the silk fibroin is efficiently secreted from the posterior silk gland as an elementary unit, 2.3 MDa protein complex, comprising six sets of a disulfide-linked H-fibroin–L-fibroin heterodimer and one molecule of fibrohexamerin/P25 [18]. In the caddisfly *S. marmorata*, Smsp-1 forms a large complex with Smsp-2, Smsp-3, and Smsp-4 as previously reported [5]. In addition, the present study suggests that the Smsp-4 oligomer potentially assembles a fundamental complex of Smsps with several sets of a disulfide-linked Smsp-1–Smsp-3 heterodimer.

In the present study, we successfully performed cDNA cloning, gene expression analysis, and recombinant protein expression of Smsps including the unusual novel proteins, Smsp-2 and Smsp-4. These results provide new molecular information and insights into a relatively unexplored field of aquatic silk proteins.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.07.041.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2014.02.002.

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