Research Article

Structure and Identification of Solenin: A Novel Fibrous Protein from Bivalve *Solen grandis* Ligament

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Fibrous proteins, which derived from natural sources, have been acting as templates for the production of new materials for decades, and most of them have been modified to improve mechanical performance. Insight into the structures of fibrous proteins is a key step for fabricating of bioinspired materials. Here, we revealed the microstructure of a novel fibrous protein: solenin from *Solen grandis* ligament and identified the protein by MALDI-TOF-TOF-MS and LC-MS-MS analyses. We found that the protein fiber has no hierarchical structure and is homologous to keratin type II cytoskeletal 1 and type I cytoskeletal 9-like, containing “SGGG,” “SYGSGGG,” “GS,” and “GSS” repeat sequences. Secondary structure analysis by FTIR shows that solenin is composed of 41.8% β -sheet, 16.2% β -turn, 26.5% α -helix, and 9.8% disordered structure. We believe that the β -sheet structure and those repeat sequences which form “glycine loops” may give solenin excellence elastic and flexible properties to withstand tensile stress caused by repeating opening and closing of the shell valves in vivo. This paper contributes a novel fibrous protein for the protein materials world.

1. Introduction

Fibrous proteins have attracted much attention for a long time because of their excellent tensile strength, elastic properties, biocompatibility, and implication for fabricating biomaterials. The most common fibrous proteins include collagen, elastin, silk fibroin, and keratin, whose microstructures, composition, primary structures, and mechanical properties have been studied in detail [1–3]. Mussel byssal thread, a fibrous biomaterial from bivalve mollusc, has also been investigated for decades. This hair-like fiber can be divided into three regions: an elastic proximal thread, stiff distal thread, and adhesive plaque regions, and it was found to contain collagenous and elastin-like domains that play a key role in the extraordinary mechanical properties [4–6]. Although these proteins have been studied in detail; however, the knowledge about fibrous proteins in bivalve ligaments is still limited.

Bivalve ligament is an elastic calcified structure which connects two shell valves dorsally and functions like a coil spring to open the valves when adductor muscles relax.

Most of bivalve ligaments are divided structurally into an outer uncalcified protein layer and an inner calcified layer made up of aragonite and matrix proteins [7]. They have attracted the interest of materials scientists as elastic biocomposite materials with particular structure presenting excellent mechanical properties [8]. Generally, an intact ligament is composed of about 40% protein and 60% calcium carbonate [9]. These proteins have been studied for decades. Earlier studies were carried out mainly on amino acid composition analysis. For instance, Kelly and Rice [10] and Kahler et al. [9] reported that ligament proteins contain high contents of glycine and methionine but are devoid of hydroxyproline and hydroxylysine. Later, Kikuchi et al. [11] confirmed the results and identified two components desmosine and isodesmosine acting as cross-link in ligament protein. More recent studies have revealed insights into gene cloning and secondary structure analysis of the rubber-like protein: abduction of scallop and the synthetic peptides inspired by abduction [12, 13]. Besides, few studies have been given to proteins, especially fibrous proteins in other bivalve ligaments. Recently, we found two novel fibrous proteins from *Siliqua radiata*

FIGURE 1: Optical photos and SEM image of the experiment sample. (a), (b) Optical photos of *S. grandis* and ligament fibrous protein, respectively, and (c) SEM image of the fibrous protein.

and *Solen grandis* ligaments. The former, named as K58, was found homologous to keratin type II cytoskeletal 1 [14, 15]; the latter, which has never been studied, is composed of protein fibers with diameter of about 120 nm (Figure 1) [16].

This paper aims to observe the microstructure of the fibrous protein (FP), identify it by MALDI-TOF-TOF-MS and LC-MS-MS analyses, and reveal its secondary structure by FTIR analysis.

2. Materials and Methods

2.1. Sample Preparation and Observations. *S. grandis* (Figure 1(a)) was freshly collected from Beihai city of Guangxi in southern China. After removing the soft body, ligaments were separated from shells mechanically, washed with deionized water, and air-dried. Then, we stripped away the outer layer and took an optical photo of the protein (Figure 1(b)) using a microscope equipped with a CCD camera. To obtain the SEM image (Figure 1(c)), FP was coated with gold and observed by a SEM (S-3400N, Hitachi) operated at accelerating voltage of 30 kV.

2.2. TEM Observations. To observe the detailed structure of FP, we carried out TEM observations. First, we soaked FP in 3% glutaraldehyde for 3 days and then fixed it with 1% osmic acid for 2 h. After being dehydrated gradually with ethanol-acetone solution, FP was permeated by using acetone-epoxy resin for 24 h. Then, the embedded FP was sliced into 70 nm thickness in longitudinal and transverse section with Leica UC7 ultramicrotome. Finally, the slices were stained with

uranyl acetate-lead citrate solution and observed by Hitachi H-7650 TEM with acceleration voltage of 100 kV.

2.3. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). For biochemical analysis, FP was ground into powder in liquid nitrogen. Then, 20 mg powder was treated with 7 M urea, 3% 2-mercaptoethanol, and 0.5 M NaOH solution at 65°C for 1 h. After being centrifuged at 12 000 rpm and 4°C for 20 min, we discarded the solution and treated the residue with the same conditions except NaOH solution that was diluted into 0.25 M. Then, the sample solution was obtained by centrifugation and dialyzed with a dialysis bag of 14 kDa molecular weight cutoff for three days. Dialysate was concentrated under vacuum, treated with 2D-Clean-Up Kit (GE Healthcare), and redissolved in lysis buffer.

After being quantified by Bradford assay, 26.1 μg protein sample and molecular weight standards (MW 14.4–94.0 kDa, TianGen Biotech) were applied to SDS-PAGE on a 12% separating gel using a JY 600 electrophoresis system (JunYi Technology). Electrophoresis was followed by silver staining and the gel band 1-1 (~78 kDa) in lane 1 (Figure 2) was excised for trypsin digestion.

2.4. In-Gel Trypsin Digestion and MALDI-TOF-TOF-MS Analysis. Gel band 1-1 was destained, dehydrated, and dried under vacuum. Then, gel pieces were rehydrated with 10 ng μL⁻¹ trypsin in 40 mM NH₄HCO₃ and 10% acetonitrile solution for 45 min in ice bath. After being incubated at 37°C for 16 h, peptides were extracted from the gel twice with 50% acetonitrile and 0.1% trifluoroacetic acid. The extraction was

FIGURE 2: SDS-PAGE pattern of FP (lane 1) and molecular weight standards (lane 2).

vacuum-dried, redissolved in 0.1% trifluoroacetic acid, and mixed with α -cyano-4-hydroxycinnamic acid (CHCA). The mixture was applied to a 4800 mass spectrometer (Applied Biosystems, Framingham) running in positive ion reflection mode in the mass range of 800–4000 Da. The ten most intense ions were selected for MS-MS analysis with acceleration voltage of 20 kV.

The acquired MS and MS-MS data were combined and searched against protein databases at NCBI and Swiss-Prot using the Mascot search engine with GPS Explorer Software (Applied Biosystems). Mass tolerance of 200 ppm and ± 0.3 Da for MS and MS-MS were set, and variable modifications, such as oxidation, methylation, and phosphorylation, were under consideration. Protein score calculated by the software was used for correct identification.

2.5. LC-MS-MS Analysis. FP sample solution was separated by SDS-PAGE again, and the similar gel band was excised and digested by trypsin. Digested sample was dissolved in 0.1% formic acid and desalted with 0.2% formic acid on a Zorbax 300 SB C18 peptide trap. Then, peptides were separated by a reversed phase C18 column (0.15 mm $\times$ 150 mm, Column Technology Inc.) with a linear gradient of 0–50% mobile phase A (0.1% formic acid and 84% acetonitrile) in mobile phase B (0.1% formic acid) over 60 min. Separated peptides were eluted into an LTQ linear ion trap mass spectrometer (Thermo Finnigan) equipped with a microspray source running in data-dependent mode with spray voltage of 3.4 kV at 200°C and full scan mass range of 300–1800 Da. Dynamic exclusion was enabled with a repeat count of 2 and exclusion duration of 1.5 min. The ten most intense ions in every full scan were selected automatically for MS-MS analysis.

The MS-MS spectra were searched against protein databases using SEQUEST algorithm. All SEQUEST searches were performed on Bioworks 3.2 software (Thermo Finnigan)

with following parameters: fully tryptic peptide, parent mass tolerance 1.4; peptide mass tolerance 1.5. Delta CN (≥ 0.1) and Xcorr (one charge ≥ 1.9 , two charges ≥ 2.2 , and three charges ≥ 3.75) were used as criteria for identification.

2.6. FTIR and Secondary Structure Analysis. FP powder was mounted in KBr and scanned using an FTIR (Nicolet 4700, Thermo Electron) with 64 scans and resolution of 2 cm^{-1} in the range of 4000–400 cm^{-1} . The amide I region (1700–1600 cm^{-1}) of the spectrum was curve fitted into Gaussian curve for secondary structure analysis. Curve-fitting was carried out using origin 7.5 software (OriginLab) equipped with peak fitting module (PFM) with following parameters: data preconditioning: methods 11; filters: savitzky-golay, polynomial 2; find peaks with threshold height of 0.002. We used the peak position of individual component of Gaussian curve to assign α -helix (1650–1657 cm^{-1}), β -sheet (1612–1640 cm^{-1}), β -turn (1655–1675 cm^{-1}), and disordered (1640–1651 and 1670–1697 cm^{-1}) structure [17, 18]. Peak area of each component was calculated to obtain secondary structure data.

3. Results and Discussion

3.1. TEM Observations. The morphology of FP has been observed by SEM in Figure 1(c) and previous study [16]. However, owing to the limited resolution of SEM, it is hard to observe the fine structure of FP, especially the detailed structure of transverse sections of the protein fibers. By using TEM, we found that transverse sections of these protein fibers are roundish with diameters ranging from 130 to 200 nm, and they arrange orderly connecting one by one like many strings of pearls (Figure 3(a)). It is worth noting that diameters of protein fibers in TEM image are larger than those in SEM (about 120 nm). This inconsistency is very likely due to the pretreatment of FP for TEM observation. At higher magnification (Figure 3(b)) and from longitudinal view (Figures 3(c) and 3(d)), we did not find any microfibrils within these protein fibers, which means that these fibers have no hierarchical structure and they are not constructed by microfibrils. This structure feature is different from keratin and collagen fibers, both of which have complex hierarchical structures and are assembled by microfibrils as elementary building blocks [1, 3]. These results imply that FP may be a new kind of fibrous protein.

3.2. MALDI-TOF-TOF-MS Results and Data Analysis. In previous study [14], we have found that FP has strikingly high Gly, Asp, Met, and Phe contents but contains trace amount of Hyp. That means that FP is not collagen. To identify this unique fibrous protein, mass spectrometry was performed and the results are shown in the following.

As expected, no proteins of bivalve mollusc were matched when searched against protein databases. However, unexpectedly, FP was identified as *Pan troglodytes* keratin type II cytoskeletal 1 (K1) and *Pongo abelii* keratin type I cytoskeletal 9-like (K9) with protein score of 625 and 491, respectively,

FIGURE 3: TEM observation of FP. (a) Transverse section of FP, (b) enlarged view of transverse section of FP that shows no microfibrils within the protein fibers, (c) longitudinal section of FP, and (d) enlarged view of longitudinal section of FP that shows no microfibrils within the protein fibers.

representing 100% confidence. Furthermore, some interesting repeat sequences of “SGGG,” “SYGSGG,” “GS,” and “GSS” were highly matched (Tables 1 and 2).

Although FP was highly matched with K1 and K9, it does not mean that FP is K1 or K9, because the molecular weight of FP (~78 kDa) (Figure 2) is higher than K1 (~64 kDa) and K9 (~63 kDa) [19, 20]. As known, K1 and K9 are fibrous proteins belonging to keratin family. In terms of structure, both of them can be divided into three domains: a highly conserved central rod domain acting as basic structural framework, an N-terminal head, and a C-terminal tail domain that are diverse in different proteins [21]. Of the matching peptides in Tables 1 and 2, 70% and 55% belong to conserved central rod domains of K1 and K9, respectively. These high matching rates imply that FP contains conserved domains similar to K1 and K9, and it should be the differences of head and tail domains that distinguish FP from K1 and K9. Therefore, we consider that FP is a novel fibrous protein homologous to K1 and K9. Since it was found from bivalve *S. grandis* ligament and identified for the first time, we named it solenin.

3.3. LC-MS-MS Results and Data Analysis. LC-MS-MS analysis shows the same results as MALDI. Similarly, no proteins were matched when searched against mollusc protein

database, but K1 and K9 were matched with 56.7% and 64.2% sequence coverage, respectively. These results again indicate that solenin is homologous to K1 and K9. Interestingly, we also found that a matrix protein from inner layer of *S. grandis* ligament has a high homology with K1, which gives us an important implication; that is, solenin is likely to assemble from matrix proteins (consider for publication elsewhere). It is known that matrix proteins from bivalve shells can control the formation of biominerals [22]. Excitingly, we have found that solenin can control pure aragonite formation at ambient condition in vitro, which suggests that solenin may serve as template for fabricating of biocomposite materials.

LC-MS-MS results also matched with repeat sequences mentioned (Tables 1 and 2). These sequences, with many repetitive “G,” are different from those of other fibrous or elastic proteins, such as “GGFGGMGGGX” of abduction [12], “GAGAGS,” “APGVGV,” and “GPGGG” of silk fibroin, elastin, and mussel byssal thread, respectively [23, 24]. However, repeat sequences of “GS” and “GSS” of solenin are identical to those of Lustrin A, an extracellular matrix protein from mollusc *Haliotis rufescens* shell [25]. These glycine- and serine-rich repeats previously had been found to form rubber-like “glycine loops” in Lustrin A and keratins to give them elastic and flexible properties [25, 26]. Such

TABLE 1: Search result of FP identified by MALDI-TOF-TOF-MS, matching keratin, type II cytoskeletal 1 [*Pan troglodytes*], NCBI database ID: gi|160961491.

Calculated mass	Observed mass	$\pm$ ppm	Position	Peptide sequence ^{a,b}
973.5312	973.572	42	396–403	IEISELNR
1033.516	1033.5568	39	479–487	TLLEGEESR
1065.5211	1065.5497	27	351–359	AQYEDIAQK
1092.5028	1092.5533	46	596–609	GSGGGSSGGSIGGR
1179.6005	1179.6487	41	372–381	YEELQITAGR
1277.71	1277.7668	44	468–478	LALDLEIATYR
1302.7151	1302.7572	32	339–350	SLDLSIIAEVK
1340.6692	1340.719	37	360–371	SKAEAESLYQSK
1393.7322	1393.7858	38	273–284	TNAENEFVTIKK
1475.7489	1475.8197	48	207–218	WELLQQVDTSTR
1475.7853	1475.8197	23	195–206	FLEQQNQVLQTK
1539.7836	1539.8457	40	456–467	LLRDYQELM*NTK
1638.8599	1638.9277	41	181–194	SLNNQFASFIDKVR
1657.7928	1657.8588	40	13–29	SGGGFSSGSAGIINYQR
1708.7131	1708.7843	42	46–65	FSSCGGGGGSFGAGGGF GS R
1716.8511	1716.9194	40	413–427	QISNLQQSISDAEQR
1993.9825	1994.0464	32	618–637	SSGGSSSVKFVSTTYSQVTR
2150.0776	2150.1382	28	219–235	THNLEPYFESFINNLR
2383.9517	2384.0327	34	514–544	GGGGGGY GS GGSS YGS GGGS YGS GGGGGGGR
3312.3079	3312.3784	21	545–583	GSYGS GGSS YGS GGGS YGS GGGGGGHGS YGS GGSSSGGYR

^aM*: oxidized methionine.^bBold peptides are the repeat sequences.TABLE 2: Search result of FP identified by MALDI-TOF-TOF-MS, matching keratin, type I cytoskeletal 9-like [*Pongo abelii*], NCBI database ID: gi|297716097.

Calculated mass	Observed mass	$\pm$ ppm	Position	Peptide sequence ^{a,b}
913.4084	913.4358	30	238–244	MTLDDFR
982.4337	982.4608	28	35–46	FSS SGGGGGGG GR
1060.5634	1060.5917	27	229–237	TLLDIDNTR
1074.5215	1074.5509	27	5–13	QFSSSYLSR
1157.5909	1157.6195	25	255–265	QGVDAIDINGLR
1232.5978	1232.6311	27	14–29	SGGGGGGGLGSGGSIR
1235.5287	1235.556	22	47–59	FSSSSGYGG GSSR
1323.6725	1323.6975	19	245–254	IKFEM*EQNLR
1867.9219	1867.9476	14	326–340	TLNDM*RQEYEQLIQAK
1966.0604	1966.0665	3	379–395	HGVQELEIELQSQLSKK
2705.1609	2705.1836	8	64–95	GGGGSFYSGGG SGGG FSASSLGGGFGGGSRGGGG SGFY SYGGG SGGG FSASSLGGGFGGG SR

^aM*: oxidized methionine.^bBold peptides are the repeat sequences.

loops should also be present in solenin as it is homologous to keratin and contains “GS” and “GSS” repeats. Besides, phenylalanine (F) and tyrosine (Y) residues are involved in these repeat domains (Tables 1 and 2); their aromatic side chains interaction will contribute to the formation of “glycine loops” (Figure 4). These loops may give solenin excellence elastic and flexible properties to withstand tensile stress caused by repeating opening and closing of the shell valves in vivo.

3.4. FTIR Spectrum and Secondary Structure Analysis. FTIR spectrum (Figure 5(a)) of solenin has been discussed in our previous study [16]. Here, secondary structure of solenin was analyzed by curve-fitting of amide I band of the spectrum, since the band is frequently used for secondary structure analysis [27, 28]. Based on previous studies [17, 18] and the curve-fitting results (Figure 5(b)), peaks of Gaussian curve were ascribed to β -sheet, β -turn, α -helix, and disordered structure, respectively. Quantitative analysis results indicate

FIGURE 4: Schematic diagram of formed (a) and unfolded glycine loops (b). Blue hexagons represent benzene rings and red dot lines represent the interaction of benzene rings side chains of phenylalanine and tyrosine.

FIGURE 5: FTIR spectra of solenin. (a) Original spectrum and (b) Gaussian line shapes of amide I region, 1700–1600 cm^{-1} ((a) adapted from Figure 7(b), [16], with permission).

that solenin is mainly composed of β -sheet structure (41.8%), with 16.2% β -turn, 26.5% α -helix, and a small amount of disordered structure (9.8%). This high β -sheet content contradicts K1 and K9 (mainly with α -helix structure), which confirms that solenin is not K1 or K9. It also implies that the head or tail domain of solenin is mainly composed of β -sheet structure since its central rod domain is similar to K1 and K9. This secondary structural feature is similar to silk [29, 30] and K58 [14]. It may endow solenin with high performance of tensile strength, just like silk; the strong fibrous protein having high tensile strength of 0.6 GPa [31] is mainly made up of β -sheet structures.

Although the tensile strength of solenin has not been determined yet for the lack of instrument, there is no doubt that solenin is subjected to repeating tensile stress with frequent closing of shell valves in vivo [16]. This implies that solenin must have superior tensile strength to withstand long-term successive rapid movement of shell valves to accommodate the rapid burrowing life habit of this species [16, 32]. In addition, solenin presents excellent solvent resistance property similar to K58 [14], which implies that the protein may serve as a good template for fabricating of biomaterials.

4. Conclusions

Solenin is a novel fibrous protein homologous to K1 and K9. It is not constructed by microfibrils and is different from collagen. Its high content of β -sheet structure (41.8%) may endow solenin with excellent tensile strength and solvent resistance property. Insight into the structure of this intriguing natural fibrous protein will provide a new template for fabricating of bioinspired materials.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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