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Purification and characterization of fibroin from the tropical Saturniid silkworm, *Antheraea mylitta*

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Abstract

The fibroin protein isolated from the posterior silkgland of the tropical Saturniid silkworm *Antheraea mylitta*, was solubilized in lithium dodecyl sulfate and purified by gel filtration. The major fraction from gel filtration was analyzed by SDS–PAGE under non-reducing and reducing conditions. One major protein band of ca 395 kDa was obtained under non-reducing conditions and a doublet band of ~197 kDa under reducing conditions. The appearance of a single spot in two-dimensional electrophoresis confirmed the purity of the protein indicating that it may be a homodimeric protein of two similar sized polypeptides. Amino acid composition analysis showed that, like other Saturniid fibroins, it is rich in glycine, alanine and serine amino acids. N-terminal amino acid sequence shows significant homology with other *Antheraea* species. The enzymatic deglycosylation analysis indicates that the fibroin protein is glycosylated and the oligosaccharides are O-linked to the protein backbone by *N*-acetylgalactoseamine moiety which conforms to a Core 1 mucin-type glycosylation pattern.

Keywords: Antheraea mylitta; Fibroin; Two-dimensional electrophoresis; Homodimer; Deglycosylation; O-glycosylation

1. Introduction

Commercially important silks are produced by lepidopteran insects of the family Bombyciidae and Saturniidae. Fibroin, the major silk protein, is best characterized in the domesticated silkworm *Bombyx mori*, a representative of Bombyciidae. *B. mori* fibroin consists of heavy (H) and light (L) chain polypeptides of 391.367 kDa (Zhou et al., 2000) and 25 kDa (Kikuchi et al., 1992) respectively, linked by a disulfide bond at the C-terminus of these two subunits (Tanaka et al., 1999). The H-chain is a fibrous protein of 5263 residues and characteristically rich in glycine (46%), alanine (30%) and serine (12%) amino acids. The bulk of the H-chain polypeptide consists of a low-complexity region (4754 residues) and is made up of 12 GX dipeptide repeat motifs (X is Ala in 65%, Ser in 23% and Tyr in 10%) separated by 11 nearly identical boundary sequences (Zhou et al., 2000). On the other hand, the L-chain polypeptide is non-fibrous and contains relatively high amounts of leucine, isoleucine, valine and acidic amino acids (Shimura, 1983). In addition, another 25 kDa polypeptide, named P25 associates with the H–L complex primarily by hydrophobic interactions (Tanaka et al., 1999). The interactions between H- and L-chains or P25 are essential for the secretion of *B. mori* fibroin protein (Takei et al., 1987; Tanaka et al., 1999). The *B. mori* fibroin is N-glycosylated and contains a small quantity of glucosamine and mannose (Sinohara et al., 1971).

The other major silk is produced by the Saturniid silkworms including *Antheraea* and *Philosamia*. From the purified high molecular weight RNAs of *Antheraea yamamai*, *A. pernyi* and *Philosamia cynthia ricini*, the estimated molecular weights of fibroin polypeptides is calculated to be 250, 220 and 230 kDa, respectively (Tamura and Sakate, 1988). The common characteristic of these fibroins is the predominance of alanine, glycine and serine amino acids. The deduced amino acid sequence of the fibroin protein of *A. pernyi* shows stretches of polyalanine repetitive motifs (Yukuhiro et al., 1997). However, to date there is no report on glycosylation and its pattern of *Antheraea* fibroins. In this paper, we report the purification and characterization of fibroin from the tropical Saturniid silkworm, *Antheraea mylitta*.

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2. Materials and methods

2.1. Preparation of fibroin from larval silkglands

The fifth instar larvae of *A. mylitta* were dissected and posterior silkglands were excised. The posterior silkglands were opened in cold distilled water and the translucent viscous material was extruded out. The extruded insoluble materials were solubilized with 1% lithium dodecyl sulfate (LDS) in 10 mm Tris–HCl, pH 8. The solubilized silkgland protein was dialysed against several changes of Buffer A. (5 M urea, 0.02 M Tris-HCl, pH 8.0) and was stored at −20°C.

2.2. Analysis of crude silkgland protein and its purification

2.2.1. Gel filtration

Gel filtration of the solubilized silkgland proteins was carried out at room temperature using Sephadex G-200 (Pharmacia) packed in a mini column (10 cm×1 cm). The column was equilibrated and run with buffer A. Several fractions were collected and O.D. was measured at 280 nm. The major peak fractions were pooled and concentrated by lyophilization.The protein content was determined by the method of Lowry et al. (1951).

2.2.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Discontinuous 5% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (1970). About 0.2 μg of the major protein from gel filtration was incubated with sample loading buffer containing 10 mM 2-mercaptoethanol at 37°C for 30 min. A similar amount (0.2 microgram) of 2-mercaptoethanol-treated and untreated samples were loaded on a 3% stacking gel cast at the top of a 5% SDS polyacrylamide gel and electrophoresed. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (Sigma) and destained in destaining solution (5% methanol, 7% acetic acid in water).

2.2.3. Two-dimensional electrophoresis

Two-dimensional electrophoresis was performed according to the method of O'Farrell (1975) which in the first case was isoelectric focusing and the second case was SDS–PAGE. Isoelectric focusing was carried out in glass tubes of 2.0 mm inner diameter using 2% Bio-Lyte pH 3–10 ampholines (Biorad) for 9600 volthours. After isoelectric focusing, the gel was equilibrated for 10 min in a buffer containing 10% glycerol, 50 mM dithiothreitol, 2.3% SDS and 0.0625 M Tris (pH 6.8) and placed at the top of a stacking gel on a 5% SDS–PAGE slab gel. Electrophoresis was carried out at 12.5 mA/gel for about 4 h. The gel was stained with Coomassie Brilliant Blue, destained and dried.

2.3. Determination of amino acid composition

The purified fibroin protein was resolved by 5% preparative SDS–PAGE under non-reducing condition as described previously. It was then electroblotted to polyvinylidene difluoride (PVDF) membrane as described by Towbin et al. (1979). The membrane was stained in Coomassie Blue R-250 and then washed thoroughly to remove any bound glycine to the membrane. For the determination of the amino acid composition of fibroin, PVDF immobilized fibroin was hydrolyzed in 6N HCl/1% phenol for 24 h at 110°C. The amino acids were analyzed by Beckman 6300 Amino Acid Analyzer at the Protein Core Lab, University of Texas, USA.

2.4. N-terminal amino acids and internal peptide sequencing

PVDF immobilized fibroin protein was sequenced by automated Edman degradation on Applied Biosystems 475A Sequencer at the Protein Core Lab, University of Texas. For determining the internal peptide sequence, the PVDF blotted protein was digested with trypsin, the peptides were isolated by reverse phase HPLC, and among them four peptides were chosen randomly and sequenced.

The N-terminal and internal peptide amino acids of the fibroin protein were compared in SWISS PROT database using the BLAST search for the sequence homology with all other protein sequence entries in SWISS PROT.

2.5. Enzymatic deglycosylation analysis

Purified lyophilized fibroin was dissolved in 1% LDS buffer and divided into four 10 μl aliquots at a concentration of 20 ng/μl each. Aliquot 1 was not deglycosylated. Aliquot 2 was incubated at 37°C for 1 h with 0.02 U NANase II (Biorad) and 0.002 U *O*-glycosidase (Biorad) in 250 mM sodium phosphate, pH 6.0. After this incubation pH of the sample was adjusted to 8.0 with a pH adjustment buffer (Biorad) of 0.5 M sodium phosphate and was further incubated at 37°C with 0.005 U PNGase F (Biorad) for 3 h. Aliquot 3 was incubated with 0.02 U NANase II at 37°C for 1 h followed by 0.005 U PNGase F at 37°C for 3 h while aliquot 4 was incubated with a combination of 0.02 U NANase II and 0.002 U *O*-glycosidase at 37°C for 1 h. The efficiency of deglycosylation in these aliquots (2, 3 and 4) was compared with non-deglycosylated aliquot 1 on a 5% SDS polyacrylamide gel. Bovine fetuin was used as a positive control of deglycosylation reactions.

3. Results

3.1. Purification and molecular weight estimation of fibroin

The fibroin protein present in the posterior silkgland of *A. mylitta* was difficult to solubilize but could be solubilized in 1% lithium dodecyl sulfate containing buffer. The solubilized proteins after dialysis in 5 M urea, 0.02 M Tris–HCl pH 8.0 (buffer A), was subjected to Sephadex G 200 gel filtration chromatography in the same buffer. The major protein fractions came out in the void volume and some minor proteins appeared in other fractions.

Electrophoretic analysis of the protein obtained in the void volume from gel filtration was carried out on 5% SDS polyacrylamide gel under non-reduced (2-mercaptoethanol untreated) and reduced (2-mercaptoethanol treated) conditions. In the non-reduced condition, the gel resolved a single band just below the stacking gel (Fig. 1 Lane b) while in reduced condition its electrophoretic mobility increased and showed a band lower than that of non-reduced protein (Fig. 1 Lane c).

The molecular weight of the protein bands observed in polyacrylamide gel (non-reduced and reduced) was determined by plotting the relative mobility of protein bands with respect to the standard molecular weight marker run in the same gel (Fig. 1, Lane a). The molecular weight of non-reduced and reduced fibroin protein bands were calculated to be ca 395 and 197 kDa, respectively.

The purity of the purified fibroin protein was determined by two-dimensional electrophoresis. Purified fibroin

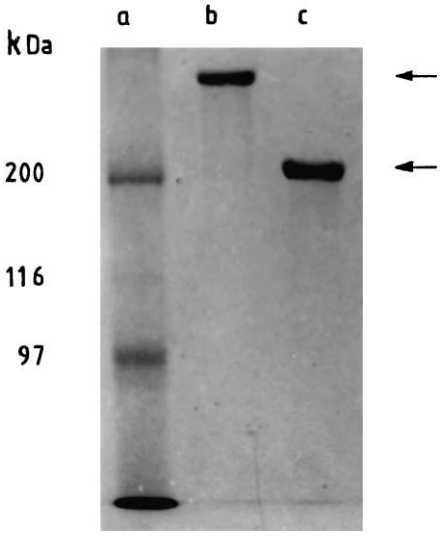

Fig. 1. SDS–PAGE analysis of fibroin from *A. mylitta*. Protein was analyzed under non-reduced (lane b) and reduced (lane c) conditions. Arrows indicate the position of fibroin. The protein molecular weight standards (Boehringer) in lane a are indicated by the numbers on the left.

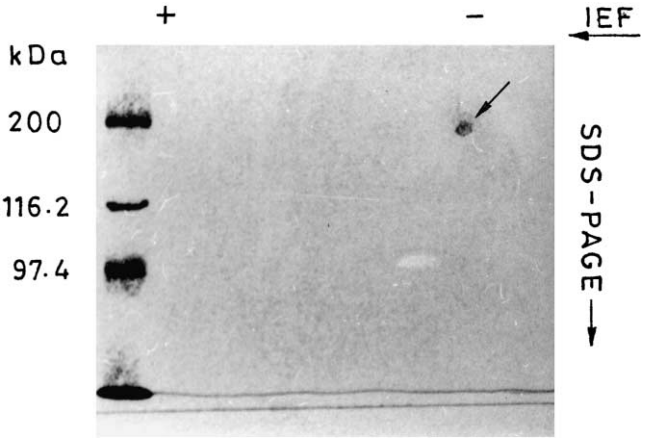

Fig. 2. Two-dimensional gel electrophoresis of purified fibroin. Arrow indicates the position of the fibroin protein. Protein molecular weight standards (Boehringer) are indicated by the numbers on the left.

was resolved in the first dimension by isoelectric focusing followed by separation on the basis of molecular weight by 5% SDS–PAGE. A single spot in the second dimension gel confirmed the purity of fibroin (Fig. 2).

3.2. Molecular characteristics of fibroin

The amino acid composition of *A. mylitta* fibroin is shown in Table 1. There is a predominance of structurally smaller amino acids residues: glycine; alanine; and serine. Among the structurally large amino acid residues, there is a much higher content of tyrosine than any other determined aromatic residue phenylalanine. Of the dicarboxylic acids, the presence of aspartic acid and asparagine combined was greater than glutamic acid and gluta-

Table 1
Amino acid composition of *A. mylitta* fibroin[a]

Amino acids	Mole (%)
Arg	3.4
Glx[b]	1.26
Ser	13.72
His	1.36
Gly	30.89
Thr	3.4
Ala	27.7
Pro	0.05
Tyr	9.37
Val	1.57
Met	0.05
Ile	0.58
Leu	0.79
Phe	0.21
Lys	0.26
Asx[c]	5.39

[a] Trp was destroyed and Cys was not determined.
[b] Glx, glutamic acid and glutamine.
[c] Asx, aspartic acid and asparagine.

Table 2
N-terminal amino acid sequence comparison among Saturniid silkworm fibroins[a]

A. mylitta		– K N I **H H D E Y V D** S **H G Q L V E R F T**
A. pernyi	***MRVIAFVILCCALGYATA***	K N L R **H H D E Y V D** N **H G Q L V E R F T**
A. yamamai	***MRVTAFVILCCATQYATA***	N N L R **H H D E Y V D** N **H G Q L V E R F T**
		* * * * * * * • * * * * * * * * *

[a] The sequences are aligned using Clustal W (Thompson et al., 1994). Identical amino acids are indicated with asterisks and similar amino acid with a dot. Residues in bold are conserved among the species of *Antheraea* silkworms. The amino acid sequence information of *A. mylitta* is obtained by automated Edman degradation while that of *A. pernyi* and *A. yamamai* is deduced from nucleic acid sequence reported by Sezutsu and Yukuhiro (2000) and Tamura et al. (1987), respectively. The amino acids in the signal peptide is indicated by italic and the putative signal peptidase recognition site –A–X–A– (Gierasch, 1989) is underlined.

mine. Among the basic amino acids, the arginine content exceeded that of lysine and histidine.

The N-terminal amino acid sequence was determined from purified fibroin blotted onto a PVDF membrane. The sequence of the first 20 amino acids is given in Table 2. A search through the GenBank and protein sequence database confirmed that the purified protein was fibroin. The purified protein, however, did not show any homology with N-terminal amino acids of the *B. mori* fibroin but showed significant homology with N-terminal sequence of Saturniid silkworm fibroin of *A. pernyi* and *A. yamamai* (Table 2). The three random tryptic digested internal peptide sequences of *A. mylitta* fibroin also showed significant homology with *A. pernyi* fibroin (Table 3).

3.3. Deglycosylation of fibroin

PNGase F is a potent endo-glycosidase which releases all asparagine linked (N-linked) oligosaccharides from glycoproteins. NANase II releases α2–3 and α2–6 linked *N*-acetyl neuraminic acid from complex oligosaccharides, while *O*-glycosidase releases unsubstituted Gal (β1,3) GalNAc (α1) disaccharide attached to serine or threonine.

Incubation of aliquot 2 with NANase II and *O*-glycosidase followed by incubation with PNGase F resulted in a shift of mobility of the fibroin when analysed on 5% SDS–polyacrylamide gel (Fig. 3 Lane b). Aliquot 3 which was incubated with NANase II and PNGase II, however, showed no shift in mobility (Fig 3 Lane c). Aliquot 4 which was incubated with a combination of NANase II and *O*-glycosidase resulted in an identical increase in electrophoretic mobility as seen in aliquot 2 (Fig. 3 Lane d). An increase in deglycosylating enzyme concentration or a longer incubation time did not effect any significant change in observed result.

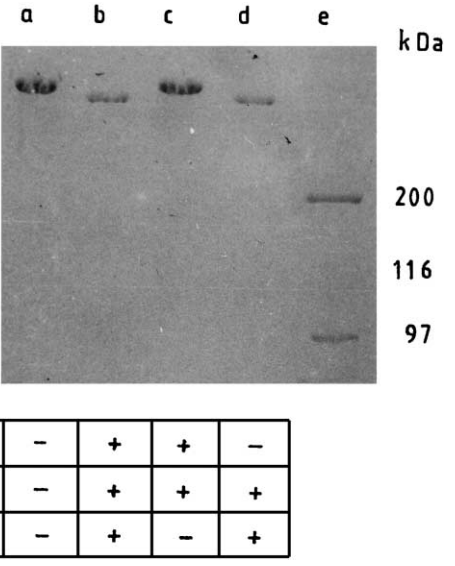

Fig. 3. Enzymatic deglycosylation analysis of *A. mylitta* fibroin by SDS–PAGE. Lane a, non-deglycosylated control; lane b, deglycosylation of fibroin with NANase II, *O*-glycosidase and PNGase F; lane c, incubation of fibroin with NANase II and PNGase F has no effect on gel mobility; lane d, increased gel mobility when incubated with NANase II and *O*-glycosidase; lane e, protein molecular weight marker (Boehringer) indicated by the numbers on the right.

4. Discussion

In the present work, we purified and characterized the fibroin protein of tropical Saturniid silkworm, *A. mylitta*. The fibroin was difficult to solubilize because the pro-

Table 3
Amino acid sequence of fibroin internal peptides and comparison with *A. pernyi* fibroin (Sezutsu and Yukuhiro, 2000) deduced amino acid sequence[a]

	Peptide 1	Peptide 2	Peptide 3
A. mylitta	LVETIVLEE	NI–HHDEYVD	NAATRPHLS
A. pernyi	^{60}LVETIVLEE^{68}	^{20}NLRHHDEYVD^{29}	^{47}NAATRPHLS^{55}

[a] The superscript numbers indicate amino acid residue number in *A. pernyi* sequence.

teins in the posterior silkgland aggregated and precipitated very quickly upon exposure to salt. To overcome this problem, the posterior silkglands were excised in cold distilled water and solubilized in LDS buffer. Although the protein could also be dissolved in 8M urea, it tended to aggregate when stored at 4°C. On the contrary no aggregation of fibroin protein was observed on storage when the fibroin protein was solubilized in LDS buffer.

Since *B. mori* fibroin consists of H and L chain polypeptides of 391.367 and 25 kDa, respectively, linked by a disulfide bond, we treated the native *A. mylitta* fibroin with 2-mercaptoethanol to reduce S–S bonds. This resulted in the disappearance of the major high molecular weight polypeptides and the appearance of polypeptides whose sizes were about half the size of the native fibroin protein (Fig. 1). We, however, did not detect any other smaller peptides by running a higher percentage of SDS-polyacrylamide gels. This suggests that the fibroin protein of *A. mylitta* is probably a homodimer and composed of two similar-sized polypeptides with an estimated molecular weight of 197 kDa, each linked by disulfide bond. Two-dimensional electrophoresis of the purified fibroin showed a single spot (Fig. 2), confirming the purity of the protein.

A comparison of the predominant amino acid residues glycine, alanine and serine among fibroins (Table 4) showed that unlike other Saturniid fibroins of *A. pernyi* and *A. yamamai*; the fibroin of *A. mylitta* has a higher glycine content than alanine. The other amino acids which are more prevalent in *A. mylitta* fibroin (such as tyrosine, glycine, threonine, serine, valine and isoleucine) than in *A. pernyi* fibroin may compensate for the lower alanine content. From the alignment of the N-terminal amino acid sequence of Saturniid silkworm fibroin using Clustal W (Thompson et al., 1994), we find that the residues are highly conserved among the three species of *Antheraea*. Moreover, there is consensus in the signal sequences between *A. yamamai* and *A. pernyi* fibroins. As shown in Table 2, in both *Antheraea* species there is a 18 residue signal peptide with a putative A–T–A signal peptidase recognition site (Gierasch, 1989). The *A. mylitta* fibroin N-terminal sequence information is obtained by Edman degradation of mature fibroin protein and hence its signal peptide sequence was not determined.

The electrophoretic gel mobility shift of the fibroin protein when treated with a combination of deglycosylating enzymes NANase II, *O*-glycosidase and PNGase F showed that the *A. mylitta* fibroin is glycosylated. When the native fibroin protein was treated with NANase II and PNGase F, there was no electrophoretic mobility shift. However, an increase of gel mobility was analysed when the native protein was treated with NANase II and *O*-glycosidase. This result indicated that the fibroin does not contain N-linked oligosaccharide but contains *O*-glycans. *O*-glycosidase and NANase II removes all Ser/Thr linked Gal (β1,3) GalNAc (α1) and all sialic acid substituted Gal (β1,3)L GalNAc (α1). Modifications of the Ser/Thr linked Gal (β1,3) GalNAc core other than by neuraminic acid (such as galactose, GlcNAc or fucose substitutions) inhibit *O*-glycosidase cleavage. This confirms that in *A. mylitta* the fibroin protein is O-glycosylated and conforms to a Core 1 mucin-type oligosaccharide structure (Van den Steen et al., 1998). The core structures are defined by the monosaccharides and the linkage attached to the first GalNAc residue, of which eight have been identified to date. In comparison to this fibroin, the oligosaccharides in *B. mori* fibroin are attached by glucosaminyl asparagine linkage (Sinohara et al., 1971).

From our findings, we conclude that *A. mylitta* fibroin is a homodimeric protein of two similar sized polypeptides of 197 kDa approximately, predominantly rich in glycine, alanine and serine amino acids. The N-terminal amino acids show a similarity with fibroins of other *Antheraea* species. The protein is *O*-glycosylated and the oligosaccharides are linked to the protein backbone by *N*-acetylgalactoseamine moiety.

Table 4
Comparison of predominant amino acids (in mole %) among fibroins

	A. mylitta	*A. pernyi*[a,b]	*A. yamamai*[c]	*B. mori*[d]	*Nephila clavipes*	
				(H chain)[a]	Spidroin 1[a,e]	Spidroin 2[a,f]
Glycine	30.89	27.28	27.5	45.86	41.85	30.55
Alanine	27.70	43.08	46.5	30.27	25.56	20.79
Serine	13.72	11.25	13.5	12.09	5.17	10.08

[a] Amino acid composition from the deduced amino acid sequence.
[b] Sezutsu and Yukuhiro (2000).
[c] Tamura et al. (1987).
[d] Zhou et al. (2000).
[e] Xu and Lewis (1990) and Beckwitt and Arcidiacono (1994).
[f] Hinman and Lewis (1992).

Acknowledgements

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