

Further Evidence for Importance of the Subunit Combination of Silk Fibroin in Its Efficient Secretion from the Posterior Silk Gland Cells

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Abstract. A locus responsible for the *Nd-s* mutation of the silkworm, *Bombyx mori*, has been mapped very close to or within the fibroin light (L) chain gene on the 14th chromosome (Takei, F., K. Kimura, S. Mizuno, T. Yamamoto, and K. Shimura, 1984, *Jpn. J. Genet.*, 59:307–313). A strain of *B. mori* carrying the homozygous *Nd-s^D* mutation (*Nd-s^D/Nd-s^D*; *Nd-s^D* is allelic to *Nd-s*) secretes <0.3% of fibroin into the lumen of the posterior silk gland compared with a strain carrying the homozygous wild-type alleles (+/+). The small amount of fibroin that is secreted in the *Nd-s^D/Nd-s^D* strain consists of the heavy (H) chain only and lacks the L chain, although the L chain mRNA and the proteins that are cross-reactable with the anti-L chain serum are present in the posterior silk gland

cells. In the hybrid silkworm, *Nd-s^D/+*, the H chain derived from either the *Nd-s^D* or + allele forms disulfide linkage with the L chain derived from the + allele and these fibroins are secreted into the lumen with an equal efficiency, but the L chain derived from the *Nd-s^D* allele remains in the cell unbound to the H chain. Some evidence suggesting structural abnormality of the L chain derived from the *Nd-s^D* allele is presented. These results, together with the previous results on the effect of the H chain gene-linked *Nd(2)* mutation (Takei, F., F. Oyama, K. Kimura, A. Hyodo, S. Mizuno, and K. Shimura, 1984, *J. Cell Biol.*, 99:2005–2010), strongly suggest that the H-L subunit combination of silk fibroin is important for its efficient secretion.

SILK fibroin produced by the silkworm, *Bombyx mori*, is composed of one heavy (H)¹ chain of ~350 kD and one light (L) chain of ~25 kD, which are connected by disulfide bond(s) (7, 18, 19). The H chain is a fibrous protein and characteristically rich in glycine, alanine, and serine (20). On the other hand, the L chain is nonfibrous and contains relatively high amounts of leucine, isoleucine, valine, and acidic amino acids (20). Genes for the H and L chains are expressed coordinately in the posterior silk gland cells (12), although they are located on different chromosomes (4, 10, 11).

A polypeptide of ~25 kD, P25, and its mRNA have also been detected in the posterior silk gland (3). However, amino acid composition of the P25, deduced from its genomic sequence (2), is significantly different from those of the fibroin L chains isolated from posterior silk glands and cocoons: particularly in the contents of Gly, Ala, Phe, Thr, Pro, Lys, His, Arg, and Cys (19, 20). It has been reported that small polypeptides associated with fibroin consist of one major and at least one minor component (7, 19) that have slightly different electrophoretic mobilities, and we presume that the P25 corresponds to a minor component. Functional and evolu-

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1. *Abbreviations used in this paper:* H, heavy; L, light.

tionary relationships between the L chain in this study and the P25 have not been established.

A large amount of silk fibroin is synthesized in the posterior silk gland cells during the fifth instar and secreted into the lumen of the posterior silk gland and is stored there in a liquified state until it is spun out at the end of the fifth instar. Mechanisms of the efficient intracellular transport, secretion, and maintenance of the liquified state of the large fibrous protein both in the cell and lumen of the posterior silk gland have not been elucidated.

Recently we have proposed that the H-L structure is advantageous for the secretion of fibroin, based on the finding that the combination of H and L chains is not attained in a mutant silkworm, *Nd(2)/Nd(2)*, whose phenotypes are extremely low level production of fibroin and underdevelopment of the posterior silk gland (23).

In the present study, we analyzed the effect of another mutation, *Nd-s^D*. The *Nd-s^D* mutation was induced by chemical mutagenesis and has been fixed as a homozygous *Nd-s^D/Nd-s^D* strain (8). Phenotypes of the strain are similar to those of the *Nd(2)/Nd(2)* strain, although *Nd-s^D* and *Nd(2)* loci have been assigned to different chromosomes; i.e., *Nd(2)* is closely linked with *Fib-H* (a gene for the fibroin H chain) on the 25th chromosome (4, 10, 23) and *Nd-s^D* is closely linked with *Fib-L* (a gene for the fibroin L chain) on the 14th chromosome (22).

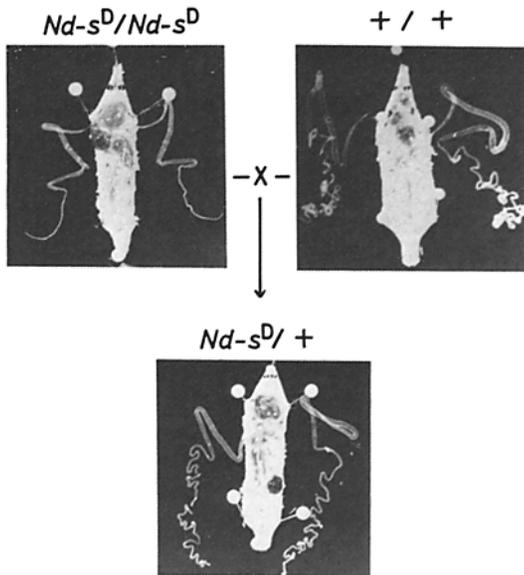


Figure 1. Appearances of the silk gland, at the fifth day of the fifth instar, of *B. mori* strains having the allelic combinations of $Nd-s^D/Nd-s^D$, $Nd-s^D/+$, and $+/+$. The + allele is the one in the haploid genome of the J-131 strain.

In this paper, we report that the small amount of fibroin secreted into the lumen of the posterior silk gland of the $Nd-s^D/Nd-s^D$ strain consists of the H chain only and lacks L chain, although the L chain gene is expressed in the posterior silk gland cell. However, in the hybrid silkworm, $Nd-s^D/+$, the H chain derived from the allele of the $Nd-s^D$ strain combines with the L chain from the allele of the + strain and is secreted into the lumen of the posterior silk gland as efficiently as the H chain from the + allele bound to the L chain from the + allele. Some evidence indicating the structural abnormality of the L chain derived from the $Nd-s^D$ allele is presented.

Materials and Methods

Larvae of *Bombyx mori*

The larvae having the genotypes of $Nd-s^D/Nd-s^D$, $Nd-s^D/+$, and $+/+$ (J-131 and Tamanashikasuri strains) were kindly supplied by Dr. T. Yamamoto of the Department of Silkworm Breeding, the Sericultural Experiment Station at Kobuchizawa, Yamanashi Prefecture, Japan. Appearances of the silk glands of the strains having different genotypes are shown in Fig. 1.

Determination of the Amounts of DNA, RNA, and Secreted Fibroin in the Posterior Silk Gland

Five pairs of posterior silk glands from the larvae at the fourth day of the fifth instar were homogenized in 5 ml of 0.02 M Tris-HCl (pH 7.5), 0.05 M KCl, 0.05 M EDTA, 0.5% SDS containing 2.5 mg proteinase K (Merck & Co., Inc., Rahway, NJ) in a Dounce-type glass homogenizer. The homogenate was subjected to the sodium perchlorate extraction and total nucleic acids were precipitated with the ethanolic sodium perchlorate reagent according to Lizardi and Engelberg (14). The precipitate was dissolved in 2 ml of 0.3 N KOH and incubated at 37°C for 18 h. The solution was neutralized with 6 N HCl and made 5% with respect to perchloric acid and stood at 0°C for 30 min. After low speed centrifugation, the supernatant was saved for quantitation of RNA, and the precipitate was suspended in 2 ml of 5% perchloric acid and DNA was hydrolyzed at 100°C for 15 min. Concentrations of RNA and DNA were determined from the absorbance at 260 nm.

Fibroin secreted into the lumen of the posterior silk gland was separated

Table I. Amounts of DNA, RNA, and the Secreted Fibroin per Pair of Posterior Silk Glands of Silkworm Strains Having Different Allelic Combinations

Allelic combination	DNA		RNA		Fibroin secreted	
	Weight	Relative ratio	Weight	Relative ratio	Weight	Relative ratio
	μg		μg		mg	
$+/+^*$	170	100	2640	100	29.5	100
$Nd-s^D/+$	84	49	1480	56	17.5	59
$Nd-s^D/Nd-s^D$	18	11	339	13	<0.1	<0.3

* The + allele represents the one in the Tamanashikasuri strain.

Each analysis was made using five pairs of posterior silk glands from the larvae at the fourth day of the fifth instar.

from tissue proteins as described in the previous paper (23) and its dry mass was determined.

Gel Electrophoresis of Fibroin

Fibroin secreted into the lumen of the posterior silk gland was isolated, either as described in the previous report (reference 23; for the experiment shown in Fig. 6) or after carefully removing the surrounding tissue with forceps from the posterior silk gland, which had been fixed in 60% ethanol (for the experiment shown in Fig. 2). The fibroin was dissolved in 60% LiSCN and dialyzed against 0.02 M Tris-HCl (pH 7.4) containing 1% SDS and 5 M urea (SDS buffer). To prepare tissue (or cellular) proteins from the posterior silk gland, the posterior silk gland was placed at -10°C for 12 h to denature fibroin secreted into the lumen, then homogenized in the SDS buffer. The homogenate was filtered through four layers of gauze to remove the coagulated fibroin. The filtrate was boiled for 10 min and subjected to SDS-PAGE according to Laemmli (13). A disk gel (0.4×5 cm or 0.3×8 cm) was used to analyze the H chain and a slab gel ($20 \times 20 \times 0.04$ or $10 \times 8 \times 0.1$ cm) was used to analyze the L chain. Two-dimensional SDS PAGE in Laemmli's buffer system (13) was carried out using the disk gel for the first dimension and the slab gel for the second dimension.

Northern Blot Hybridization

RNA was extracted from the posterior silk gland according to Feramisco et al. (6). RNA samples from different strains were denatured with glyoxal and dimethylsulfoxide according to Thomas (24) and subjected to 1% agarose-gel electrophoresis in 0.012 M Tris, 6 mM sodium acetate, 0.3 mM EDTA (pH 7.5). After electrophoresis, the gel was stained with 1 $\mu\text{g}/\text{ml}$ ethidium bromide. RNAs in the gel were transferred to a nylon membrane filter (Gene Screen; New England Nuclear, Boston, MA) in 0.025 M sodium phosphate (pH 6.8) for 20 h, and the filter was baked at 80°C for 2 h. The RNA filter was prehybridized in 0.05 M Tris-HCl (pH 7.4), 1 M NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10 \times Denhardt's solution, 10% dextran sulphate, 100 $\mu\text{g}/\text{ml}$ sheared, denatured salmon sperm DNA at 65°C for 7 h, then hybridized with two to three times 10^5 cpm/ml ^{32}P -labeled L chain cDNA clone, pLA23, labeled by the multiprime labeling method of Feinberg and Vogelstein (5), at 65°C for 18 h. The filter was washed twice in 2 \times SSC (1 \times SSC = 0.15 M NaCl, 0.015 M $\text{Na}_3\text{-citrate}$, pH 7.0), 0.5% SDS at 65°C for 30 min each, twice in 3 \times SSC at 65°C for 30 min each, dried, and exposed to an x-ray film (Kodak XS-1) with an intensifying screen at -80°C .

Protein-blotting Analysis

After SDS-PAGE, proteins were transferred electrophoretically to a sheet of nitrocellulose membrane and were reacted with the anti-fibroin L chain serum (17), followed with ^{125}I -labeled protein A (Amersham International, Amersham, United Kingdom) according to Burnette (1). Autoradiography was performed as described above.

Extraction of Fibroin from SDS Polyacrylamide Gel

A portion of the SDS polyacrylamide gel corresponding to the fibroin band was cut out, minced with a razor blade, and extracted with the SDS buffer by shaking at 37°C overnight. Gel fragments were removed by passing the mixture through a layer of glass wool packed into a disposable tip of a pipette

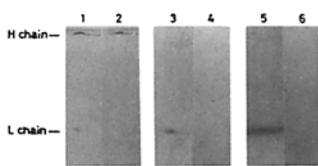


Figure 2. Analysis of the H-L subunit combination of the secreted fibroin. Fibroins secreted into the lumens of the posterior silk glands of *Nd-s^D/Nd-s^D* and *+/+* (J-131) strains were subjected to 4% SDS-

PAGE without reduction (left to right), and reduced in the SDS buffer containing 5% 2-mercaptoethanol and separated by 12.5% SDS-PAGE (top to bottom). Only the region of the gel where the secreted fibroin migrated in the first dimension is shown. The gel was stained with Coomassie Brilliant Blue R-250 (lanes 1 and 2) or subjected to electroblotting, reactions with anti-L chain serum and ¹²⁵I-protein A, and autoradiography (lanes 3–6). (Lane 1) Fibroin (13 µg) of the *+/+* (J-131) strain; (lane 2) fibroin (13 µg) of the *Nd-s^D/Nd-s^D* strain; (lane 3) fibroin (4 µg) of the *+/+* (J-131) strain; (lane 4) fibroin (18 µg) of the *Nd-s^D/Nd-s^D* strain; (lane 5) fibroin (160 µg) of the *+/+* (J-131) strain; (lane 6) fibroin (180 µg) of the *Nd-s^D/Nd-s^D* strain.

under centrifugal force. Fibroin in the extract was reduced with 1% 2-mercaptoethanol and subjected to SDS-PAGE.

Results

Low Level Accumulation of Fibroin in the Lumen of the Posterior Silk Gland of the *Nd-s^D/Nd-s^D* Strain and Its Recovery in the *Nd-s^D/+* Strain

Amounts of DNA, RNA, and fibroin secreted into the lumen per pair of posterior silk glands of the silkworm strains having the allelic combinations of *Nd-s^D/Nd-s^D*, *Nd-s^D/+*, and *+/+* (Tamanashikasuri) are compared in Table I. It is noted that the amount of fibroin secreted into the lumen in the *Nd-s^D/Nd-s^D* strain was <0.3% of that in the *+/+* strain, while the amounts of DNA and RNA in the posterior silk glands of the *Nd-s^D/Nd-s^D* strain were ~10% of those in the *+/+* strain. These results indicate that synthesis and/or secretion of fibroin are markedly suppressed in the presence of the homozygous *Nd-s^D* mutation.

It is also noted that the amount of fibroin secreted in the *Nd-s^D/+* strain was more than 175 times higher than that in the *Nd-s^D/Nd-s^D* strain, although amounts of DNA and RNA per pair of posterior silk glands in the former strain were only about five times higher than those in the latter strain. The amount of fibroin accumulated in the lumen of the posterior silk gland of the *Nd-s^D/+* strain was ~60% of that of the *+/+* strain, but it contained approximately equal amounts of H chains from the *Nd-s^D* and the *+* alleles as described in the later section.

Absence of L Chain in Fibroin Secreted into the Lumen of the Posterior Silk Gland of the *Nd-s^D/Nd-s^D* Strain

To examine the H-L subunit combination of the secreted fibroin, fibroin was isolated from the lumen of the posterior silk gland and subjected to 4% SDS polyacrylamide disk gel electrophoresis as the first dimension. Proteins in the gel were reduced with 2-mercaptoethanol and subjected to 12.5% SDS polyacrylamide slab gel electrophoresis as the second dimension. The gel was either stained with Coomassie Brilliant Blue R-250 (Fig. 2, lanes 1 and 2) or subjected to electroblotting and reactions with anti-L chain serum and ¹²⁵I-protein A (Fig. 2, lanes 3–6). As shown in Fig. 2, the L chain was associated with the secreted H chain in the lu-

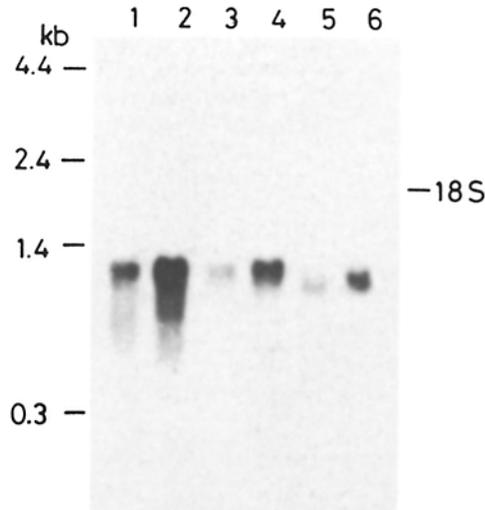


Figure 3. Detection of the fibroin L chain mRNA by Northern blot hybridization. Total RNA extracted from the posterior silk gland was subjected to electrophoresis and blot hybridization with ³²P-labeled L chain cDNA clone, pLA23. (Lane 1) RNA (2 µg) from the *+/+* (Tamanashikasuri) strain; (lane 2) as in lane 1 but with 5 µg RNA; (lane 3) RNA (2 µg) from the *Nd-s^D/+* strain; (lane 4) as in lane 3 but with 5 µg RNA; (lane 5) RNA (10 µg) from the *Nd-s^D/Nd-s^D* strain; (lane 6) as in lane 5 but with 20 µg RNA. Fragment sizes shown on the left were determined using the RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD) and the position of 18S RNA indicated on the right was determined from the mobility of the 18S rRNA in the RNA samples used.

men of the posterior silk gland of the *+/+* (J-131) strain (lanes 1, 3, and 5), but association of the L chain was not detectable with either method for the small amount of H chain secreted in the *Nd-s^D/Nd-s^D* strain (lanes 2, 4, and 6).

Detection of L Chain mRNA in the Posterior Silk Gland Cells of the *Nd-s^D/Nd-s^D* Strain

To elucidate the reason for the absence of the L chain in the small amount of fibroin secreted in the *Nd-s^D/Nd-s^D* strain, the L chain mRNA was sought in the posterior silk gland of the *Nd-s^D/Nd-s^D* strain. Total RNA preparations from the posterior silk glands of *Nd-s^D/Nd-s^D*, *Nd-s^D/+*, and *+/+* (Tamanashikasuri) strains were subjected to Northern blot hybridization with the ³²P-labeled L chain cDNA clone pLA23.

In each case, as shown in Fig. 3, the ³²P-labeled cDNA hybridized to a band corresponding to the size (~1.3 kb) of the L chain mRNA (12, 15), indicating that the L chain gene is transcribed in the posterior silk gland cells of the *Nd-s^D/Nd-s^D* strain. However, the size of the L chain mRNA produced in the *Nd-s^D/Nd-s^D* strain (Fig. 3, lanes 5 and 6) seems to be ~0.15 kb shorter than the mRNA produced in the *+/+* strain (Fig. 3, lanes 1 and 2). It is also noted that concentration of the L chain mRNA in the *Nd-s^D/Nd-s^D* strain as determined by the densitometric scanning of the autoradiogram of Fig. 3 was only ~4% of that in the *+/+* strain.

Abnormal Molecular Size of the L Chain Derived from the *Nd-s^D* Allele

Translational products of the L chain mRNA in the posterior silk gland cells of *Nd-s^D/Nd-s^D*, *Nd-s^D/+*, and *+/+* (Tama-

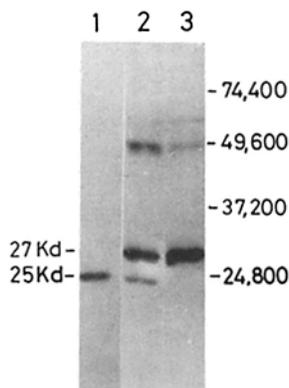


Figure 4. Radioimmunochemical detection of the fibroin L chain in the tissue protein fraction from the posterior silk gland. The tissue proteins (1 μ g/lane) were subjected to 12.5% SDS-PAGE (20 \times 20 \times 0.04 cm) after reduction with 2-mercaptoethanol. Proteins were transferred to a sheet of nitrocellulose membrane and subjected to the reaction with the anti-L chain serum, followed with 125 I-protein A, and autoradiography. The tissue protein fractions analyzed were from: (lane 1) the

+/+ strain (Tamanashikasuri, a normal producer of fibroin); (lane 2) the $Nd-s^D/+$ strain; and (lane 3) the $Nd-s^D/Nd-s^D$ strain. Molecular mass markers indicated on the right were dimer, trimer, tetramer, and hexamer of the horse heart cytochrome *c* (Oriental Yeast Co., Ltd., Tokyo, Japan). Molecular mass of the L chain was also determined using the following markers: bovine serum albumin (66,000), ovalbumin (45,000), chymotrypsinogen A (25,000), trypsinogen (24,000), and soybean trypsin inhibitor (20,000).

shikasuri) strains were examined. Total proteins from the posterior silk gland tissue were reduced with 2-mercaptoethanol and subjected to SDS-PAGE and protein-blotting analysis using the anti-L chain serum and 125 I-protein A as described in Materials and Methods. It is shown in the autoradiogram of Fig. 4 that one main band of \sim 27 kD and two weaker bands having lower mobilities were detected in the protein fraction from the $Nd-s^D/Nd-s^D$ strain (lane 3). The apparent molecular mass of the main band protein was \sim 2,000 D higher than that of the main band (\sim 25 kD) detected for the +/+ strain (lane 1). The protein fraction from the hybrid silkworm, $Nd-s^D/+$, showed two bands corresponding to the products derived from the $Nd-s^D$ and + alleles and slow moving components similar to those found in the $Nd-s^D/Nd-s^D$ strain (lane 2).

Inability of L Chain from the $Nd-s^D$ Allele to Combine with the H Chain within the Posterior Silk Gland Cells

Two-dimensional electrophoresis was used to investigate whether the L chain derived from the $Nd-s^D$ allele is able to form disulfide linkage with the H chain in the posterior silk gland cells of $Nd-s^D/Nd-s^D$ and $Nd-s^D/+$ strains. In the first dimension, tissue proteins from the posterior silk gland of the $Nd-s^D/Nd-s^D$, $Nd-s^D/+$, or +/+ (Tamanashikasuri) strain were subjected to 4% SDS-PAGE without reduction. The disk gel was then soaked in the SDS buffer containing 5% 2-mercaptoethanol and applied to the second dimension 12.5% SDS-PAGE. Detection of the L chain was carried out by electroblotting, radioimmunochemical reactions with the anti-L chain serum and 125 I-protein A, and autoradiography.

As shown in Fig. 5, the L chain that had been combined with the H chain was detectable in the posterior silk gland cells of the +/+ strain; however, all of the L chain behaved as free molecules in the case of the $Nd-s^D/Nd-s^D$ strain. In the hybrid silkworm, $Nd-s^D/+$, the L chain (27 kD) derived from the $Nd-s^D$ allele had not been combined with the H

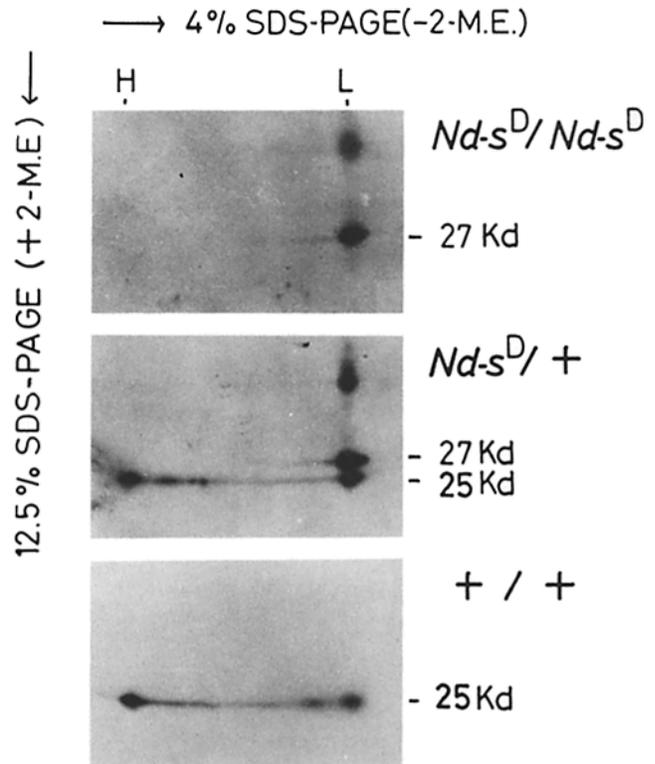


Figure 5. Two-dimensional gel electrophoresis of the tissue protein fraction from the posterior silk gland and radioimmunochemical detection of the fibroin L chain. Tissue proteins from the posterior silk gland (5 μ g for each analysis) of the $Nd-s^D/Nd-s^D$, $Nd-s^D/+$, or +/+ (Tamanashikasuri) strain were subjected to 4% SDS-PAGE (a disk gel) without reduction (first dimension), then the gel was soaked in the buffer containing 2-mercaptoethanol and subjected to 12.5% SDS-PAGE (a slab gel, 10 \times 8 \times 0.1 cm) (second dimension). The L chain in the gel was detected by the protein-blotting method, using the anti-L chain serum and 125 I-protein A, and autoradiography.

chain, but a significant fraction of the L chain (25 kD) from the + allele had been bound to the H chain.

We conclude from these results that the L chain derived from the $Nd-s^D$ allele is unable to form disulfide linkage with the H chain in the posterior silk gland cells of the $Nd-s^D/Nd-s^D$ and $Nd-s^D/+$ strains. It is unlikely that the inability to form the intermolecular disulfide linkage is caused by the absence of factor(s) required for the formation of a disulfide bond, because the L chain from the + allele was able to combine with the H chain in the same cellular milieu of the posterior silk gland of the $Nd-s^D/+$ strain. Comments on the tailing in the first dimension and the slow moving component in the second dimension in Fig. 5 are included in the Discussion.

Recovery of the Level of Secretion of the H Chain from the $Nd-s^D$ Allele in the Hybrid Strain, $Nd-s^D/+$

Fibroins produced by different strains of *B. mori* occasionally show different electrophoretic mobilities due to genetic polymorphisms of the H chain gene (9, 21). For a hybrid silkworm, $Nd-s^D/+$, in that the + allele was derived from the

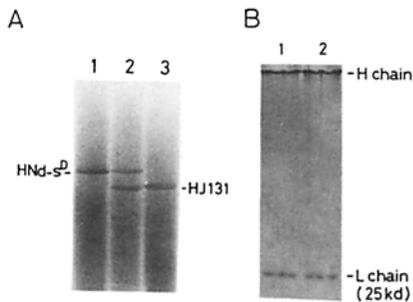


Figure 6. (A) 4% SDS-PAGE of the fibroin secreted into the lumen of the posterior silk gland. The secreted fibroins (5 μ g/lane, without reduction) were from: (lane 1) the $Nd-s^D/Nd-s^D$ strain; (lane 2) the $Nd-s^D/+$ strain; and (lane 3) the $+/+$ strain (J-131). (B) 12.5% SDS-PAGE of the fibroin extracted from each band in A, lane 2, and after reduction with 2-mercaptoethanol. (Lane 1) The fibroin containing the H chain from the $Nd-s^D$ allele; (lane 2) the fibroin containing the H chain from the $+(J-131)$ allele.

J-131 strain, fibroins secreted into the lumen of the posterior silk gland were resolved into two bands of approximately equal intensities upon 4% SDS-PAGE as shown in Fig. 6 A, lane 2. The slowly moving band corresponds to the fibroin containing the H chain from the $Nd-s^D$ allele (lane 1) and the fast moving band corresponds to the fibroin containing the H chain from the J-131 allele (lane 3).

Fibroins secreted into the lumen of the posterior silk gland of the hybrid strain, $Nd-s^D/+$ (J-131), were separated by 4% SDS-PAGE as shown in Fig. 6 A, lane 2, and the fibroin in each band was extracted, reduced with 2-mercaptoethanol, and subjected to 12.5% SDS-PAGE. As shown in Fig. 6 B, both fibroins consisted of H and L chains, indicating that the H chain derived from the $Nd-s^D$ allele, as well as the H chain derived from the J-131 allele, formed disulfide bond(s) with the L chain from the J-131 allele and was secreted efficiently.

Table I shows that the level of fibroin secreted into the lumen of the posterior silk gland in the hybrid silkworm, $Nd-s^D/+$, was $\sim 60\%$ of that in the $+/+$ strain. The results shown in Fig. 6, A and B demonstrate that the H chain derived from the $Nd-s^D$ allele binding to the L chain from the $+$ allele accounts for about half of the fibroin secreted into the lumen. The lower total level of secretion, compared with that of the $+/+$ strain, may be due to the insufficient supply of the L chain from the $+$ allele.

Discussion

In the present study, we revealed that the small amount of fibroin secreted into the lumen of the posterior silk gland of the $Nd-s^D/Nd-s^D$ mutant strain consisted of the H chain only (Fig. 2), although transcripts of the L chain gene (Fig. 3) and proteins cross-reactive with the anti-L chain serum (Fig. 4) were present within the cells of the posterior silk gland. The L chain produced from the $Nd-s^D$ allele does not combine with the H chain even in the same cellular milieu where the L chain from the normal allele can form the H-L subunit combination (Fig. 5).

Silkworms carrying the $Nd-s$ mutation form thin cocoons

consisting mostly of sericin (8). Takei et al. (22) conducted a three-point test involving U (a locus governing a pigmentation pattern of the body surface), $Nd-s$, and $Fib L$ (a gene for the fibroin L chain) on the 14th chromosome, and observed no crossing over between $Fib L$ and $Nd-s$. It is thus very likely that the $Nd-s$ mutation represents a particular type of mutation occurring in the fibroin L chain gene.

One of the abnormalities of the L chain produced from the $Nd-s^D$ allele, which is allelic to the $Nd-s$ mutation (8), is that its apparent molecular mass is $\sim 2,000$ D higher than that of the L chain produced from the $+$ allele (Figs. 4 and 5), although the size of the L chain mRNA of the $Nd-s^D/Nd-s^D$ strain seems to be ~ 150 nucleotides shorter than that of the $+/+$ strain (Fig. 3). This difference in the apparent molecular mass of the L chain is greater than the differences observed among the L chain variants caused by genetic polymorphisms of the L chain gene (11). Another abnormality of the L chain from the $Nd-s^D$ allele is the appearance of higher molecular size forms that are cross-reactive with the anti-L chain serum on the 12.5% SDS-PAGE pattern after reduction with 2-mercaptoethanol (Fig. 4). Similar higher molecular size forms are also noticeable in the pattern of the two-dimensional gel electrophoresis shown in Fig. 5, again on the second dimension gel after reduction with 2-mercaptoethanol. Relationship between the increase in the apparent molecular mass and the appearance of the higher molecular size forms after reduction with 2-mercaptoethanol is not elucidated. Some conformational change of the L chain from the $Nd-s^D$ allele might be induced after reduction, which leads to the formation of intermolecular aggregates that persist even after the treatment with SDS.

Gamo and Sato (8) conducted electron microscopic studies on the posterior silk glands of $Nd-s^D/Nd-s^D$ and $Nd-s^D/+$ strains and found that the endoplasmic reticulum was markedly enlarged, but development of the Golgi complex and fibroin secretory vesicles were undetectable in the $Nd-s^D/Nd-s^D$ strain; on the other hand, the Golgi complex was moderately developed and the fibroin secretory vesicles were present, although the endoplasmic reticulum was still fairly enlarged in the $Nd-s^D/+$ strain. From these observations they suggested that the transport of fibroin from the endoplasmic reticulum to the Golgi complex is blocked in the $Nd-s^D/Nd-s^D$ strain. If this is the case, formation of the disulfide bond(s) between the H and L chains in the endoplasmic reticulum is likely to be a prerequisite for the efficient transport of the nascent fibroin to the Golgi complex. An alternative possibility may be that accumulation of the $Nd-s^D$ L chains causes some general malfunction of the intracellular transport. However, this idea is less likely because our previous investigation on the effect of the $Nd(2)$ mutation, which is closely linked with the fibroin H chain gene on the 25th chromosome, revealed that the H chain produced from the $Nd(2)$ allele could not combine with the normal L chain in the posterior silk gland cells of both $Nd(2)/Nd(2)$ and $Nd(2)/+$ strains and that the secretion of the H chain from the $Nd(2)$ allele was markedly reduced in both strains (23).

Oyama et al. (16) have shown that the H and L chains of fibroin are synthesized on the membrane-bound polysomes in the posterior silk gland cells. They have also shown, using a cell-free protein-synthesizing system consisting of the

membrane-bound polysomes and the soluble fraction from the posterior silk gland, that elongation of both H and L chain polypeptides takes place in this system and the products are incorporated into membrane vesicles in which a substantial fraction of the nascent L chain forms disulfide bond(s) with the H chain. These results suggest that the H-L subunit combination is formed soon after the nascent H and L chains enter the endoplasmic reticulum in the posterior silk gland cell of a normal producer of fibroin. It is yet unknown if the nascent L chain binds to the growing H chain molecules. The substantial tailing of the L chain from the + allele on the 4% SDS-PAGE in the absence of 2-mercaptoethanol (Fig. 5) may suggest that the binding of the L chain to the growing molecules of H chain occurs in the posterior silk gland cells, but this point requires further investigations.

Previous results on the effect of the *Nd(2)* mutation and the present results on the effect of the *Nd-s^D* mutation together strongly support the notion that the H-L subunit combination is important for the efficient intracellular transport and secretion of the silk fibroin in the posterior silk gland of *B. mori*.

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