Reduced Level of Secretion and Absence of Subunit Combination for the Fibroin Synthesized by a Mutant Silkworm, Nd(2)

FUSAHO TAKEI, FUMITAKA OYAMA, KEN-ICHI KIMURA, AKIO HYODO, SHIGEKI MIZUNO, and KENSUKE SHIMURA
Laboratory of Biochemistry, Department of Agricultural Chemistry, Tohoku University, Sendai 980 Japan.
Dr. Shimura’s present address is Department of Biological Sciences, Tohoku Fukushi University, Sendai, 980 Japan.

ABSTRACT Fibroin is normally composed of one H chain (350 kd) and one L chain (25 kd) which are connected by disulfide bond(s). However, the small amount of fibroin secreted into the lumen of the posterior silk gland of the Nd(2) (naked pupa) mutant does not contain L chain, although L chain mRNA is present and L chain is synthesized in the posterior silk gland cells of the mutant. In a hybrid silkworm, Nd(2)/Tamanashikasuri, where Tamanashikasuri is a normal producer of fibroin, L chain from the two alleles are distinguishable electrophoretically. It is demonstrated using this system that the L chain from the Nd(2) allele can combine normally with the H chain from Tamanashikasuri and the H-L complex is secreted normally. In another hybrid system, Nd(2)/J-131, where J-131 is a normal producer of fibroin, fibroin derived from the two alleles are distinguishable due to the different electrophoretic mobility of H chain. The fibroin derived from the J-131 allele is composed of H chain and L chain, while the fibroin derived from the Nd(2) allele is devoid of L chain, and its secretion is greatly reduced. We present evidence suggesting that the H chain derived from the Nd(2) allele is structurally abnormal and discuss how the H-L subunit structure is advantageous in the secretion of fibroin.

The silk gland of silkworm, Bombyx mori, secretes two kinds of proteins, fibroin from the posterior silk gland and sericin from the middle silk gland. It has been shown that the native fibroin extracted from the lumen of posterior or middle silk gland is composed of a large and a small component connected by disulfide bond(s) (1–3). The molar ratio of the two polypeptides is one to one (3). The large component (350 kd) has an amino acid composition of so-called “fibroin” type, while the small component (25 kd) has a markedly different amino acid composition (4). In this study, we name the large component as heavy chain (H chain) and the small component as light chain (L chain). The H chain has been studied extensively from various aspects (5–8), while the L chain is poorly characterized. Recently mRNA for the L chain was identified in an in vitro translation system coupled with immunological detection with an antiserum against the L chain (9).

In the present study, we compared subunit structures of fibroin synthesized by a mutant silkworm, Nd(2), and by strains producing normal levels of fibroin (normal producers). The “naked pupa” strain (symbolized as Nd) was found as a naturally occurring mutant (10). Nd(2), one of the Nd-type strains, produces a thin cocoon that consists mostly of sericin. The posterior silk gland of Nd(2) (Fig. 1A) is extremely short in size, undeveloped, and produces little fibroin. It has been shown, however, that the number of cells present in the posterior silk gland of Nd(2) is the same as that of a normal producer (10). The middle silk gland of Nd(2) is normal and secretes a normal level of sericin. The structural gene of the H chain and the locus responsible for the Nd(2) mutation have been shown to be linked and both are present on the 25th chromosome (11). 

1 The H chain gene was originally assigned to the 23rd chromosome because of its linkage with the Nd(2) locus (11). However, location of Nd(2) has recently been corrected to be on the 25th chromosome (12). Accordingly, the H chain gene is assigned to the 25th chromosome.
In this paper, we report that the small amount of fibroin secreted into the lumen of the posterior silk gland of the Nd(2) mutant has only H chain and lacks L chain, although an apparently functional L chain is synthesized within the cells of the posterior silk gland. Evidence is presented for the synthesis of a structurally abnormal H chain by Nd(2).

MATERIALS AND METHODS

Bombyx mori Larvae: The larvae of J-131, J-138, J-139, Nd(2), Tamanashikasuri, Nd(2)/J-131, and Nd(2)/Tamanashikasuri were kindly supplied by Dr. T. Yamamoto of the Department of Silkworm Breeding, the Sricultural Experiment Station at Kobuchizawa, Yamanashi Prefecture, Japan.

Preparation of Fibroin and Tissue Proteins from the Posterior Silk Gland: The posterior silk gland of the silkworm at the fifth day of the fifth instar was excised, dried, and ground as described in Materials and Methods. A small amount of coagulated protein thread was obtained and its amino acid composition was analyzed. The amino acid composition of this material showed a typical fibroin-type pattern.

RESULTS

Low Level Synthesis and Secretion of Fibroin-type Protein in the Posterior Silk Gland of Nd(2)

First it was examined whether fibroin is synthesized in Nd(2). The posterior silk glands of Nd(2) at the fifth day of the fifth instar were excised, dried, and ground as described in Materials and Methods. A small amount of coagulated protein thread was obtained and its amino acid composition was analyzed. The amino acid composition of this material and of the secreted fibroin in the J-131 strain (normal producer) are compared in Table I. The protein secreted by the posterior silk gland of Nd(2) showed a typical fibroin-type amino acid composition. These data indicate that Nd(2) synthesizes and secretes a small amount of fibroin-type protein into the lumen of the posterior silk gland.

Absence of L Chain in the Fibroin Secreted into the Lumen of the Posterior Silk Gland of Nd(2)

To detect L chains in the fibroins secreted, fibroins prepared from the lumen of the posterior silk glands were subjected to SDS PAGE according to Laemmli (14). The quantity of fibroin H chain was determined by densiometric scanning of the stained gels using a Beckman spectrometer ACTA III (Beckman Instruments, Palo Alto, CA).

Amino Acid Analysis: Amino acid composition of the protein was determined with a Hitachi 835 amino acid analyzer after hydrolysis with 6 N HCl at 110°C for 24 h in an evacuated tube.

Preparation of RNA: RNA was extracted from the posterior silk glands according to the method of Lizardi and Engelberg (15). The extracted RNA was precipitated in 2 M LiCl at 4°C to remove DNA. The LiCl step was usually repeated twice.

Northern-blot Hybridization: RNA was denatured essentially as described by McMaster and Carmichael (16). The glyoxylated RNA was subjected to 1% agarose gel electrophoresis and transferred from the gel to a nitrocellulose membrane filter in 20 x SSC [SSC = 0.15 M NaCl-0.015 M Na2 citrate, pH 7.0] according to Thomas (17). Prehybridization was carried out as described by Wahl et al. (18), except that 250 μg/ml of sonicated, denatured chicken DNA was included. Then the heat-denatured 32P-labeled L chain cDNA clone pL23 was added and hybridization was carried out at 42°C for ~20 h. The pl23 was labeled with [α-32P]dCTP (Amersham Corp., Arlington Heights, IL) by nick-translation. Specificity of pL23 was tested by hybridization-translation assay (Kimura, K., F. Oyama, H. Veda, S. Mizuno, and K. Shimura, in preparation). The filter was washed in 0.2 x SSC at 50°C and exposed to Kodak Ortho G film at -70°C using a Kodak intensifying screen.

Protein-blotting Analysis: SDS PAGE was performed according to the method of Laemmli (14). Transfer of proteins from the gel to a sheet of nitrocellulose membrane was made electrophoretically. Blotting of antibody to the nitrocellulose-immobilized proteins and reaction with 125I-labeled protein A (Amersham Corp.) were performed according to Burnette (19). The anti-L chain serum was obtained from the rabbit immunized with the purified fibroin L chain and its specificity was proved by a solid phase radioimmunoassay using 125I-protein A (20).

Extraction of Fibroin from SDS Polyacrylamide Gel: A portion of SDS polyacrylamide gel corresponding to the fibroin band was sliced and extracted with 0.01 M sodium phosphate buffer (pH 7.0) containing 5 M urea and 1% SDS by shaking at 37°C overnight. The fibroin in the extract was reduced with 1% 2-mercaptoethanol and subjected to SDS PAGE.

Gel Electrophoresis of H Chain mRNA: Fibroin H chain mRNA was analyzed in a 0.6% agarose-1.6% acrylamide gel containing 52% formamide, 40 mM triethanolamine, 1.5 M formaldehyde, and 1.3 mM EDTA (pH 7.5). Prior to loading, samples were denatured in 58% formamide containing 40 mM triethanolamine, 1.5 M formaldehyde, and 1.3 mM EDTA (pH 7.5) by heating at 60°C for 5 min.

Southern-blot Hybridization: Genomic DNAs from different strains were digested with EcoRI plus XhoI, electrophoresed on a 0.7% agarose gel, transferred to a nitrocellulose membrane, and subjected to the hybridization with 32P-labeled fibroin H-chain mRNA from Bombyx mori, as described by Manning and Gage (6).
TABLE I

| Amino acid | Nd(2)  | J-131 |
|------------|--------|-------|
| Gly        | 43.2*  | 42.9  |
| Ala        | 29.5   | 30.0  |
| Ser        | 9.7    | 12.2  |
| Tyr        | 4.1    | 4.8   |
| Val        | 2.5    | 2.5   |
| Asp        | 1.9    | 1.9   |
| Glu        | 1.8    | 1.4   |
| Thr        | 1.2    | 0.9   |
| Ile        | 0.7    | 0.6   |
| Leu        | 0.7    | 0.6   |
| Phe        | 1.7    | 0.7   |
| Pro        | 0.6    | 0.5   |
| Met        | 0.2    | 0.1   |
| Cys        | 0.05   | 0.03  |
| Lys        | 0.7    | 0.4   |
| His        | 0.2    | 0.2   |
| Arg        | 0.5    | 0.5   |

* Figures are expressed as mole percent.

12.5% SDS PAGE (the percentage indicates concentrations of acrylamide plus bis-acrylamide) after reduction with 2-mercaptoethanol. As shown in Fig. 2, L chain was detected in the fibroin from the normal producer (J-131), however no L chain could be detected in the fibroin from Nd(2). These results indicate that the fibroin secreted into the lumen of the posterior silk gland of Nd(2) is composed of only H chain and lacks L chain.

Production of a Functionally Normal L Chain from the Nd(2) Allele

To answer the question of whether L chain is synthesized in the cells of the posterior silk gland of Nd(2), mRNA for the L chain was first looked for in the posterior silk gland of Nd(2). Total RNA prepared from the posterior silk gland of Nd(2) or J-131 (normal producer) was subjected to Northern-blot hybridization with 32P-labeled L chain cDNA clone pLA23. In both cases the 32P-labeled cDNA hybridized to a single band corresponding to the size of L chain mRNA (Fig. 3, lanes 1 and 2), indicating that L chain mRNA is present in the posterior silk gland cells of Nd(2) as well as in those of J-131. However, concentration of the L chain mRNA in Nd(2) was ~20% of that in J-131, judging from the densitometric scanning data for Fig. 3. The reason for this observation is unknown at the moment.

The translational product of L chain mRNA was next looked for in the posterior silk gland cells of Nd(2). Total proteins of the posterior silk gland tissue were reduced with 2-mercaptoethanol and subjected to the protein blotting analysis using anti-L chain serum and 125I-protein A. An autoradiography shown in Fig. 4A, lane I revealed that the anti-L chain serum reacted with one polypeptide band having an apparent molecular weight of 25,000, which corresponded to that of L chain.

These results demonstrate that the L chain is synthesized in the posterior silk gland cells of Nd(2) but it is undetectable in the lumen of the silk gland. There are two possible explanations for the absence of L chain in the secreted fibroin of Nd(2): a structural defect of the H chain or the L chain that prevents the formation of disulfide bond(s), or the absence of factor(s) required for the formation of disulfide bond(s).

Recently three forms (A–C) of genetic variants of the L chain have been detected electrophoretically in our laboratory (21). Utilizing this polymorphism, it was attempted to see whether the L chain derived from the Nd(2) allele in a hybrid silkworm was able to bind with the H chain derived from the normal allele and be secreted into the lumen of the posterior silk gland.

L chain phenotypes, present in the cells of the posterior silk glands of Nd(2), Tamanashikasuri (a normal producer), and Nd(2)/Tamanashikasuri, were investigated by SDS PAGE and the protein blotting analysis. As shown in Fig. 4A, the L chain in the cells of Nd(2)/Nd(2) showed A type (lane 1), the L chain in the cells of Tamanashikasuri/Tamanashikasuri...
showed C type (lane 2), and in the posterior silk gland cells of Nd(2)/Tamanashikasuri, A and C types of L chain were present with the ratio of approximately one to one (lane 3). When the fibroin secreted into the lumen of the posterior silk gland of the hybrid Nd(2)/Tamanashikasuri, was subjected to 12.5% SDS PAGE after reduction with 2-mercaptoethanol, two bands of L chain (A and C types) were detected with the ratio of approximately one to one (Fig. 4B, lane 3). The Nd(2)/Nd(2) strain secreted a small amount of fibroin with no detectable L chain (Fig. 2, lane J and Fig. 4B, lane J). In the hybrid, Nd(2)/Tamanashikasuri, almost all of the H chain secreted into the lumen of the posterior silk gland was the type from the Tamanashikasuri allele (data not shown), just as seen in the case of Nd(2)/J-131 (Fig. 5B, lane 2; see below).

These results indicate that the L chain from the Nd(2) allele is functionally normal and can combine with the H chain from the Tamanashikasuri allele and be secreted normally.

Subunit Structure and Secretion of Fibroin in a Hybrid Silkworm, Nd(2)/J-131

In a hybrid silkworm, Nd(2)/J-131, obtained from the cross between Nd(2) and J-131 (a normal producer), the posterior silk gland develops a little better than in Nd(2)/Nd(2) (Fig. 1C).

The tissue materials of the posterior silk glands and the fibroin secreted into the lumen of the hybrid, Nd(2)/J-131, were separated as described in Materials and Methods. The electrophoretic profiles of the proteins in these two fractions after reduction with 2-mercaptoethanol on a 12.5% SDS polyacrylamide gel are shown in Fig. 5A. The tissue proteins consisted of heterogeneous components, whereas the secreted fibroin fraction consisted of fibroin H and L chains. The same protein materials (reduced with 2-mercaptoethanol) were subjected to 4% SDS PAGE to examine the fibroin H chain region more carefully (Fig. 5B). The ratios of the H chain derived from the Nd(2) allele (H Nd(2)) to the H chain derived from the J-131 allele (H J-131) were determined densitometrically; H Nd(2)/HJ-131 was 0.53 for the tissue proteins and <0.01 for the secreted fibroin (Fig. 5C). These results indicate that secretion of H Nd(2) is reduced to a level <2% of that of H J-131.

Next, it was examined whether L chains were present in the fibroins derived from the Nd(2) and the J-131 alleles within the posterior silk gland cells of the hybrid, Nd(2)/J-131. Fibroins derived from the two alleles were separated and extracted from the 4% SDS polyacrylamide gel (Fig. 6A). These fibroins were reduced with 2-mercaptoethanol, and subjected to electrophoresis on 12.5% SDS polyacrylamide gel. As shown in Fig. 6B, the fibroin derived from the J-131 allele contained L chain, but L chain was undetectable in the fibroin from the Nd(2) allele. In this system, L chains from the J-131 and Nd(2) alleles are indistinguishable, as both L-chains show A type (see Fig. 4) of electrophoretic mobility.

These results indicate that the absence of L chain in the fibroin of Nd(2) is likely caused by the failure of the formation of disulfide bond(s) between H and L chains due to some structural defect of the H chain. It is unlikely that the formation of the H-L subunit structure is prevented by the absence of an independent factor responsible for the formation of disulfide bonds, because the H and L chains from the J-131 allele are associated and secreted normally in the same cellular milieu.

Structural Abnormality of the H Chain of Nd(2)

Fig. 7A shows 4% SDS PAGE profiles of H chains prepared from the fibroins secreted into the lumen of the posterior silk glands of three normal producers (J-131, J-139, and J-138) and Nd(2). The H chains from each of the three normal
FIGURE 6 Analysis of the H-L subunit structure of fibroins from the Nd(2) and the J-131 alleles in a hybrid silkworm, Nd(2)/J-131. Tissue proteins of the posterior silk gland were subjected to 4% SDS PAGE without treating with 2-mercaptoethanol, and fibroins from the Nd(2) and the J-131 alleles were extracted separately from the gel. (A) Fibroins (3 μg each) thus separated were subjected to 4% SDS PAGE and stained with Coomassie Brilliant Blue. (B) Fibroins (3 μg each) from different alleles were subjected to 15% SDS PAGE after reduction with 2-mercaptoethanol and stained with Coomassie Brilliant Blue. (Lane 1) Fibroin from the J-131 allele; (lane 2) fibroin from the Nd(2) allele.

producers gave a single band having different mobilities. The fibroin prepared from Nd(2) also gave a single H chain band but had the slowest mobility among the four strains compared.

Then, molecular sizes of the H-chain mRNAs from these strains were compared by PAGE under denaturing conditions (Fig. 7 B). Molecular sizes of the H-chain mRNAs from the three normal producers were proportional to molecular sizes of their H chain (Fig. 7, A and B). The H chain mRNA from Nd(2) showed the same molecular size as that of the H chain from J-138 (Fig. 7 B). Similar results were obtained when the sizes of the EcoRI/XhoI fragment (representing nearly the entire length of the H chain structural gene [6, 22]) from these strains were compared (Fig. 7 C). The lengths of the EcoRI/ XhoI fragments were parallel, except for Nd(2), to the sizes of the H chain. The length of the fragment from Nd(2) was about the same (~15.5 kb) as that from J-138.

These results indicate that the H chain of Nd(2) has an apparently higher molecular weight than that expected from the molecular size of its mRNA or of the EcoRI/XhoI fragment of its structural gene.

DISCUSSION

In the present study, polymorphisms of the fibroin H and L chains are utilized to distinguish between different allelic products.

In Nd(2)/Nd(2), only a small amount of fibroin is secreted into the lumen of the posterior silk gland and the secreted fibroin consists of the H chain (H Nd(2)) only. This phenomenon indicates that the H-L subunit structure is not an absolute requirement for the secretion of fibroin. However, on comparing intra-cellular and secreted levels of H Nd(2) and H J-131 in a hybrid, Nd(2)/J-131, it is evident that the secretion of H Nd(2) is markedly reduced (Fig. 5). H J-131 is
secreted in combination with an L chain (Fig. 2). Thus, it seems likely that the formation of a complex between H and L chains through disulfide bond(s) facilitates the transport of H chain within and/or across the silk gland cell. An alternative possibility may be that the Nd(2) mutation causes elimination of a crucial cysteine residue from the H chain (see the discussion below) making it impossible to associate with the L chain, and at the same time disrupting an oligopeptide sequence around the cysteine residue which is essential for normal secretion. We are, however, in favor of the former possibility, because we have found recently that in another mutant silkworm, Nd-s, L chain is abnormal and only a small amount of H chain is secreted. In a hybrid, Nd-s/+ , H chain from the Nd-s allele is secreted normally combining with the L chain from the normal allele. We are now examining this system in detail, hoping to provide conclusive evidence that the H-L subunit structure is required for the efficient secretion of fibroin in the posterior silk gland.

H chain is a giant polypeptide with a molecular weight of ~350 kD and ~70% of its amino acid residues are composed of glycine and alanine. As both amino acids give a hydrophobic character to the protein, L chain might be necessary to form a certain confirmation of H chain which is required to maintain adequate solubility of H chain during its secretion. Fig. 6 shows that H Nd(2) is not combined with an L chain even within the same cellular milieu where H J-131 forms an H-L subunit structure. Thus, it is most likely that H Nd(2) has some structural abnormality that interferes with the formation of disulfide bond(s) with L chain. The Nd(2) mutation is not segregatable from the structural gene of H chain in the backcross of J-131 × (Nd(2)/J-131) (Hyodo, A., F. Takei, T. Gamo, T. Yamamoto, S. Mizuno, and K. Shimura, in preparation). These findings support the possibility that the structural abnormality introduced in the H chain is responsible for the Nd(2) phenotype. There are two possible explanations for the abnormality of H Nd(2). (a) Deletion or rearrangement involving a cysteine residue that forms a disulfide bond with L chain; (b) conformational change of H chain which interferes with the access of L chain.

One structural abnormality noticed for H Nd(2) is its apparently higher molecular weight than that expected from the size of its mRNA or of the EcoRI/XhoI fragment of its structural gene (Fig. 7). Lizardi (23) has shown that the polypeptide length of H chain generally corresponds to the molecular size of the H chain mRNA among different strains. However, we cannot explain at the moment how the absence of the H-L subunit combination and the apparently abnormal molecular weight of H Nd(2) are correlated.

It is shown in Fig. 5 that the relative ratio of H Nd(2)/H J-131 is 0.53 in the posterior silk gland cells of the hybrid, Nd(2)/J-131. Hyodo et al. (24) estimated that the ratio of H Nd(2) mRNA/H J-131 mRNA in the posterior silk gland of Nd(2)/J-131 was 0.24:1. A question of whether the lower level expression of the H Nd(2) allele is caused at the transcriptional level or by instability of H Nd(2) mRNA, is unsolved. Ueda et al. (22) have shown recently that ~2-kb sequences upstream from the 5'-end of the H chain gene are generally similar between Nd(2) and J-139 (a normal producer) except for some minor differences, which suggests the presence of small insertion, deletion, or base changes. It remains to be elucidated if the latter small differences are involved in the low level expression of the H Nd(2) gene.

At the moment, it is difficult to understand the relationship between the Nd(2) mutation and underdevelopment of the posterior silk gland in Nd(2)/Nd(2) or in Nd(2)/+. Iijima (25) has reported that the posterior silk gland cells of Nd(2) are abundant with remarkably distended endoplasmic reticulum and that Golgi apparatus and mitochondria are poorly developed. The inhibition of secretion of H Nd(2) may cause distension of endoplasmic reticulum and lead to a lower level of cellular metabolism and underdevelopment of the posterior silk gland.

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