IV. Chemical composition and biosynthesis of silk proteins

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Increasing interest has been directed to the biosynthesis of silk proteins as a model system of protein biosynthesis and its regulation in eukaryotes[1,2]. The most striking characteristic of the silk gland system is unique chemical composition and structure of the protein synthesized in the silk glands; more of this will be mentioned below. In both the posterior and middle silk glands there exists a strong correlation between tRNA species and the amino acid compositions of the silk proteins[3]. This fact has evoked our great interest in the problem of the functionally adaptive formation of tRNA and its regulation in the silk glands, and extensive work on this problem is being done at several laboratories - mainly in France, the United States and Japan.

Recently it was demonstrated that silk fibroin of *Bombyx mori* is composed of large and small subunits presumably with a ratio of 1:1[4]. The large subunit has a mol.wt of about 350,000 and an amino acid composition of the so-called 'fibroin type', that is, rich in glycine, alanine and serine. The small subunit, in contrast, is rich in acidic and hydrophobic amino acids as well as glycine and alanine. The main feature of the large subunit of fibroin is that glycine residues alternate with alanine, serine, tyrosine and other minor amino acids almost throughout its sequence. This feature of the fibroin large subunit makes it possible for us to identify nascent peptides of fibroin and also to predict the nucleotide composition and sequence of fibroin mRNA; this in turn contributes greatly to the advancement of molecular biological studies on silk formation[5].

This chapter describes the chemical characteristics of the silk proteins and their synthesis in the silk glands in connection with the spinning process of the silk proteins.

1. Chemical structure of fibroin

Sedimentation analysis of native fibroin has shown that the main sedimenting species is a 10S component corresponding to 3.7×10^5 daltons in mol.wt. As will be mentioned later, the occurrence of genetic variants of fibroin possessing different mobilities on gel electrophoresis has been reported[6]. In view of this fact, the mol.wt of fibroins of *Bombyx mori* may vary between 3.4 and 3.8×10^5 daltons among species.

Native fibroin splits into large and small subunits after treatment with thiol compounds such as 2-mercaptoethanol[7-9]. According to our data obtained from a semi-quantitative fractionation of the small subunit from cocoons of *Bombyx mori*, the molar ratio of large and small subunits was estimated to be 1:1, assuming that the mol.wts of large and small subunits are 3.5×10^5 and 2.5×10^4 daltons, respectively[4]. As shown in table 1, the amino acid composition of the large subunit exhibits a typical fibroin type, that is, it abounds in glycine, alanine and serine, whereas the compositon of the small subunit is greatly different from such a fibroin type. Digestion of the large subunit of fibroin by chymotrypsin gives in insoluble precipitate and a mixture of soluble peptides. The precipitate fractions (Cp) is composed only of glycine, alanine and serine, with a little tyrosine, and have an

Table 1. Amino acid composition[a] of silk proteins

Amino acid	Cocoon fibroin[b]	Posterior silk gland fibroin[c]			Sericin whole[d]
		Whole	Large subunit	Small subunit	
Glycine	43.7	42.9	49.4	10.0	13.5
Alanine	28.8	30.0	29.8	16.9	6.0
Serine	11.9	12.2	11.3	7.9	33.4
Tyrosine	5.1	4.8	4.6	3.4	2.6
Valine	2.2	2.5	2.0	7.4	2.8
Aspartic acid	1.3	1.9	0.65	15.4	16.7
Glutamic acid	1.0	1.4	0.70	8.4	4.4
Threonine	0.90	0.92	0.45	2.8	0.53
Phenylalanine	0.61	0.67	0.39	2.7	0.53
Methionine	-	-	-	0.37	0.04
Isoleucine	0.71	0.64	0.14	7.3	0.72
Leucine	0.51	0.55	0.09	7.2	1.1
Proline	0.31	0.45	0.31	3.0	0.68
Arginine	0.46	0.51	0.18	3.8	3.1
Histidine	0.16	0.19	0.09	1.6	1.3
Lysine	0.31	0.38	0.06	1.5	3.3
Cysteine	-	-	-	-	0.15
CM-Cysteine			-	1.6	

[a]Data are expressed as mol%. [b]Lucas et al., J. molec. Biol. *2* (1960) 339. [c]Shimura et al., J. seric. Sci. Tokyo *51* (1982) 20. [d]Komatsu, Bull. seric. Exp. Stn Japan *26* (1975) 170.

average mol.wt of about 4000, corresponding to about 60 amino acid residues. Its structure has been shown to be Gly · Ala · Gly · Ala · Gly [Ser · Gly · (Ala · Gly)n]$_8$ · Ser · Gly · Ala · Gly · Tyr where n is usually 2 and has a mean value of 2 (Lucas et al.)[10].

The soluble fraction (Cs) of the chymotryptic digest consists of various kinds of peptides which are smaller in size than the Cp peptides mentioned above[11]. The chain lengths of the Cs peptides are in the range of about 3–35 amino acid residues. The Cs peptide were fractionated into 3 groups having chain lengths of about 25 ~ 35 (group 1), 8 ~ 15 (group 2) and 3 ~ 6 (group 3) respectively, by Sephadex G-15 gel filtration. Among them, the peptides of group 1 exhibited an amino acid composition similar to that of the Cp peptides, but the chain length of group 1 peptides was about one half of the Cp peptides. Group 2 peptide was composed of essentially 2 kinds of octapeptides, that is, G-A-G-A-G-A-G-T and G-A-G-V-G-A-G-T(G, Gly; A,Ala; T,Tyr; and V,Val). Groups 1, 2 and 3 peptides occupied about 32%, 50% and 18% of a total soluble peptide recovered.

Although the construction of a whole primary structure of the fibroin large subunit is still incomplete at present, these experimental data indicate that the large subunit has a structure in which glycine residues occupy alternate positions almost throughout the whole molecule. The pattern will thus be -X-Gly-X-Gly-X-Gly-X-Gly-, where X is either Ala, Ser, Tyr, Val or some other minor amino acid.

The ratio of Cp to Cs fractions obtained from the large subunit is about 55:45 in terms of amino acid residues. Taking all these into account, together with the results observed with X-ray diffraction analysis and acid partial hydrolysis, it may be assumed that the large subunit has a copolymer-type structure in which the Cp peptide region (about 60 amino acid residues) and the Cs peptide region (a mean value of about 50 amino acid residues) alternate with each other.

Very little information on the structure of the fibroin small subunit is available at present except that it has an amino acid composition quite different from that of the large subunit, and has a mol.wt of about 25,000[4,9]. Recently, however, a messenger RNA for the small subunit was isolated from the posterior silk gland[21]. By using this mRNA, isolation of the small subunit gene and its structural analysis are under investigation.

2. *Biosynthesis of silk protein*

At the end of the last instar, the larvae of silkworms, *Bombyx mori* and other species of silk producing Lepidoptera, synthesize 2 kinds of proteins, fibroin and sericin. The fibroin is synthesized exclusively in the posterior silk gland and the sericin in the middle silk gland. These highly specified tissues provide us with a rather simple and useful system to carry out biochemical experiments. It is also advantageous to biochemical studies that we have many mutant strains of silkworms which have been selected and stocked in several laboratories of silkworm genetics[12].

a) Silk gland development and fibroin production

During larval life, silk gland cells undergo a cyclic development related to the hormonally controlled alternation of molts and intermolts. After the 4th molt, the metabolic activities of the larvae are markedly elevated and the silk gland grows almost exponentially until about the 6th day of the 5th instar. Figure 1 shows the changes of DNA, RNA and fibroin contents in the posterior silk gland during the 5th larval stage. Rapid syntheses of DNA and RNA take place in the first half of the 5th instar (fig. 1,C and D). Their maximal increments per day were observed on the 3rd day, while the synthesis of fibroin reaches its maximum on the 6th day (fig. 1,A and B). The bulk RNA given in figure 1 (D) comprises rRNA, tRNA and mRNA. Among them, the 2 former exhibit almost the same changes as the total RNA. Although these data were obtained in a preliminary study, we have reason to suspect that a sequential expression of genes concerned in protein-synthesizing machinery is precisely regulated in the silk gland at the 5th stage.

b) Adaptive tRNA formation in the silk glands

Much interest has been directed to the fact that the tRNA population in the silk gland is closely correlated with the amino acid composition of the silk protein synthesized in the gland. Figure 2 shows the changes in amino acid acceptor activities of tRNA from the posterior and middle silk glands at various stages during the 5th instar[13]. Acceptor activities of glycine, alanine and serine in the posterior silk glands (fig. 2,A) increase markedly up to the 5th day of the 5th instar, while those for other amino acids exhibited relatively small changes. As already mentioned in section 1, these 3 amino acids are found abundantly in fibroin. Similar results were obtained from the middle silk glands (fig. 2,B). In contrast to the results from the posterior silk gland, the largest increase in the acceptor activity in the middle silk gland was observed for serine, a major amino acid in sericin. Such a functional adaptation of the tRNA population in the cell to the amino acid composition of the protein being synthesized is – among the various tissues examined thus far – most striking in the silk gland. Aminoacyl-tRNA synthetase activity in the posterior silk gland also changes in a way corresponding to fibroin composition. If we take the specific activity at the 4th molt stage as a reference, the activities of glycyl-, alanyl- and seryl-tRNA synthetases increased 11, 4 and 6 times, respectively, during the 7 days in the 5th instar, whereas leucyl-tRNA synthetase activity

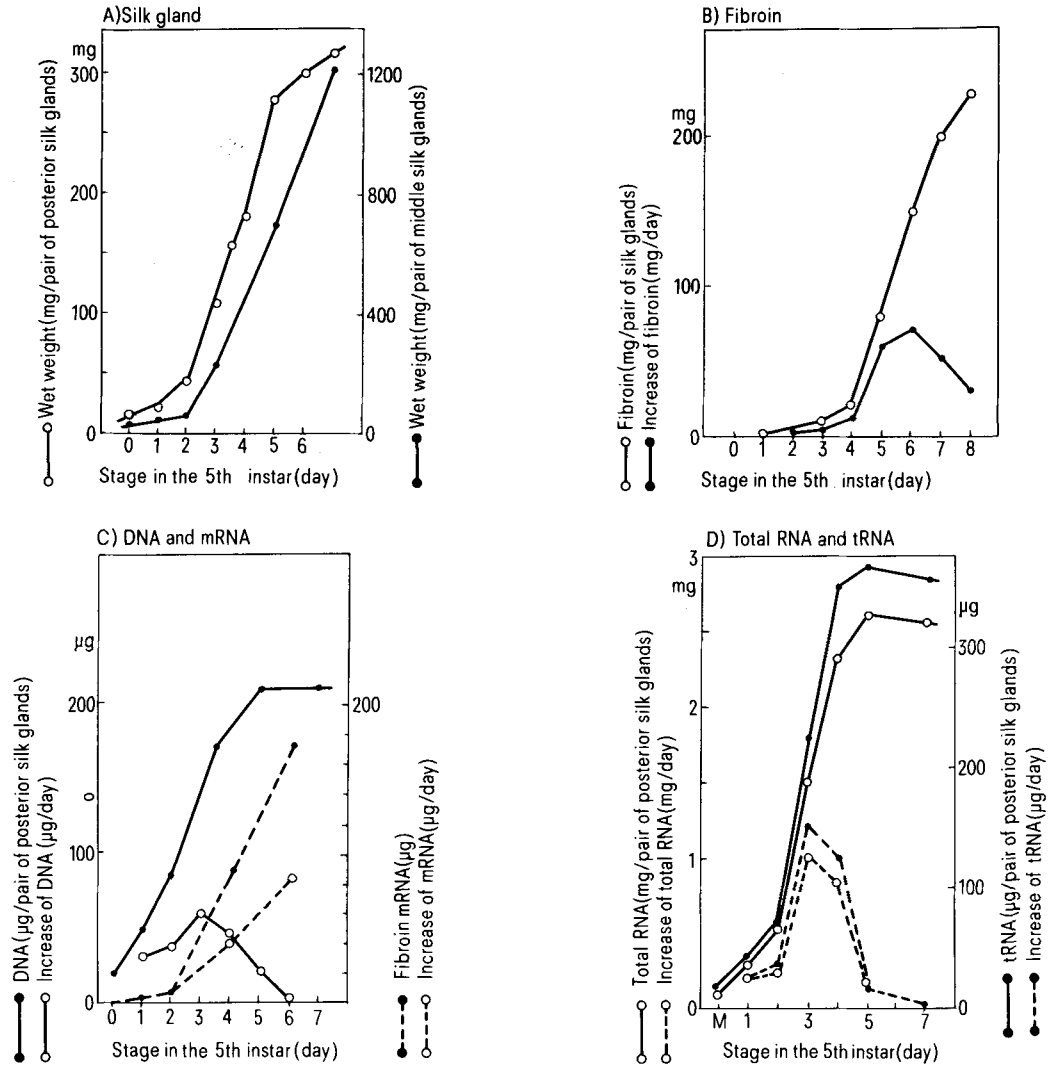


Figure 1. Changes of DNA, RNA and fibroin contents in the silk glands during the 5th larval stage of *Bombyx mori*.

decreased to some extent during the 5th instar[13]. Since K_m values of these 4 aminoacyl-tRNA synthetases for yeast tRNA's are very similar during the 5th instar[14], the changes of enzymatic activities represent quantitative changes in the intracellular concentration of synthetases and not merely a modification of the enzymatic activity. These results suggest the existence of one of the regulating mechanisms in protein synthesis at the level of tRNA and its aminoacylation. One possible mechanism for such functional adaptation may be a specific amplification of the tRNA gene. At present, however, there is no evidence to support the amplification of tRNA genes in the silk gland.

It is generally observed that tRNA is synthesized through a precursor molecule that is longer than the mature product. Separation of precursor molecules from individual tRNA species from *Bombyx mori* can be done by two-dimensional polyacrylamide gel electrophoresis[3,15]. A quantitative analysis of each tRNA precursor species demonstrates that the tRNA precursor population accumulated during a short-time incubation with ^{32}P divided roughly into 4 distinct main regions corresponding to individual precursor species to $tRNA_1^{Gly}$, $tRNA_2^{Gly}$, $tRNA_2^{Ala}$ and $tRNA^{Ser}$, respectively. Thus, it is apparent that the adaptation of tRNA population in the silk glands occurs by a preferential transcription of particular tRNA genes.

Recently, a fragment of *Bombyx mori* DNA containing a $tRNA^{Ala}$ gene was cloned and transcribed in vitro with *Xenopus* germinal vesicle extracts[16,17]. Sprague et al.[18], moreover, have carried out a homologous in vitro transcription experiment to examine the requirement for normal 5′ flanking DNA in the activity of $tRNA^{Ala}$ gene in vitro transcription. It was demonstrated that at least 2 regions of DNA are required for accurate transcription of $tRNA^{Ala}$ gene by a homologous *Bombyx mori* extract. The molecular mechanism controlling the expression of the tRNA genes, however, still remains unexplained.

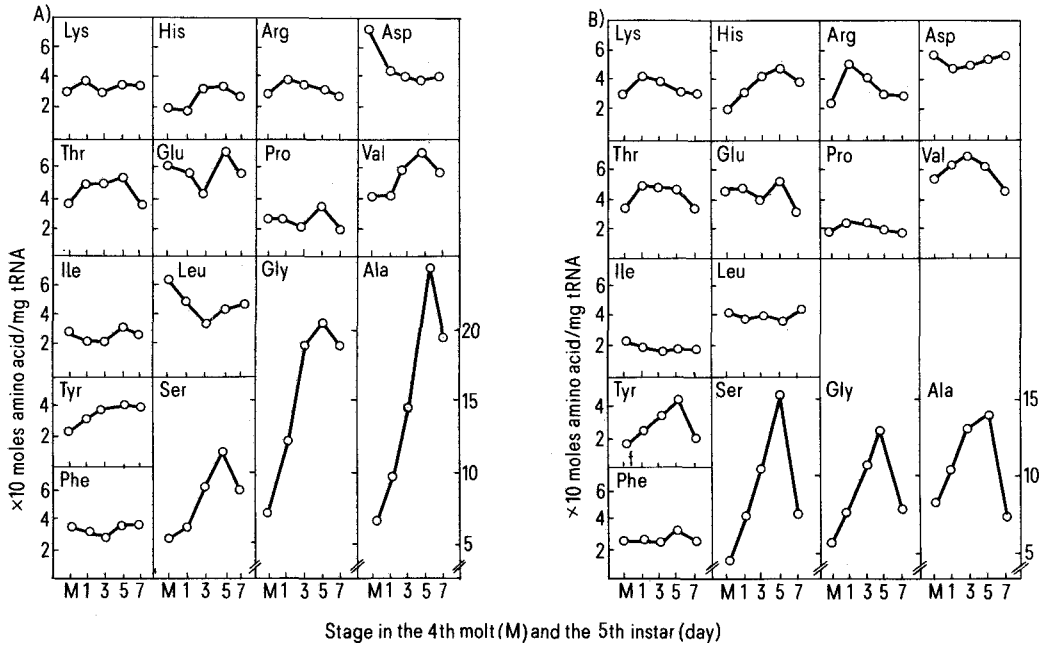


Figure 2. Changes in amino-accepting activities of tRNA from the posterior (A) and middle (B) silk glands during the 5th instar.

c) Synthesis of fibroin

As described in chapter II, fibroin is synthesized on membrane-bound polysomes. Electron microscopic observation revealed that the polysomes contain 45–112 ribosomes per mRNA molecule[19]. The intracellular pathway of the newly synthesized fibroin has been followed by electron microscopic autoradiography using in vitro pulse-chase labelling with ^{3}H-glycine[20]. The label was found successively in the rough endoplasmic reticulum, the Golgi complexes, the fibroin globules, the cell lumen border and the lumen in the gland. The mean time required for secretion of synthesized fibroin into the lumen of the gland has been estimated at about 25 min by the method of ^{14}C-glycine injection into the body cavity of the silk worm at the 5th day of the 5th instar. Although fairly detailed information is now available for each step of fibroin synthesis, only a brief account of the results on the mechanism of protein synthesis is given here.

To obtain information on the initiation and elongation of fibroin synthesis, we isolated nascent peptides from the posterior silk gland polysomes and fractionated them into 12 fractions according to their molecular size by gel filtration[21]. Figure 3 shows the relation between the contents of major amino acids and the average molecular weight of the peptides. As the average mol.wt of the peptides increases, the contents of glycine, alanine, and serine increase gradually, whereas the amounts of acidic amino acids decrease. As a result of these gradual changes, the amino acid composition of the nascent peptides becomes increasingly similar to that of fibroin as the mol.wt of the peptides increases. It is noteworthy that the nascent

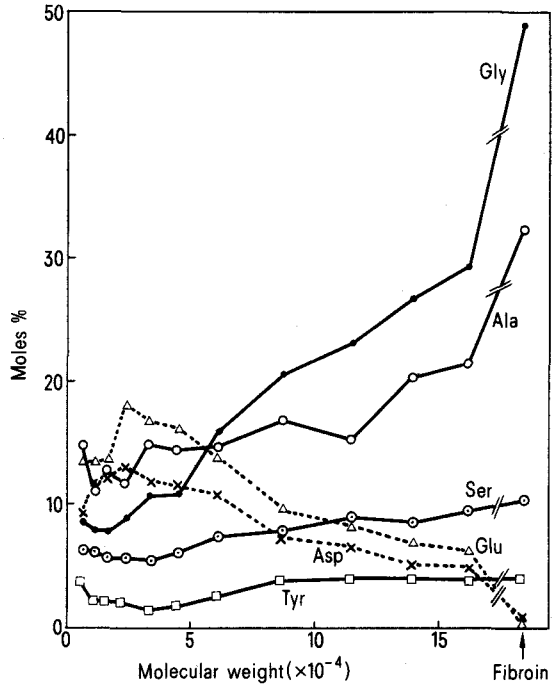


Figure 3. Changes of amino acid composition of the nascent peptide fractions from the posterior silk gland polysomes with increases of molecular size of the peptides.

peptides of smaller mol.wt (around 25,000) bear a similar amino acid composition to that of the small subunit of fibroin described in section 1.

Two possible explanations for this result may be considered: 1. At an early stage of fibroin synthesis, a peptide rich in acidic amino acids is synthesized. When the growing peptide has reached a mol.wt close

to 25,000, the amino acid sequence of the growing peptide turns to a so-called fibroin-type sequence, rich in glycine, alanine, and serine. 2. In the posterior silk glands, syntheses of a peptide rich in acidic amino acids and of a fibroin type peptide proceed individually.

Recently, Couble et al.[22] isolated a poly A containing RNA from the posterior silk gland of *Bombyx mori,* which becomes highly abundant at the period of fibroin synthesis and secretion. More recently, we have also isolated a small-size RNA (about 14S) from the posterior silk gland and assayed it for template activity in a wheat germ cell free system[23]. It has been demonstrated that the RNA serves effectively as a messenger RNA of the small subunit of fibroin. Thus, the 2nd possibility suggested above seems more likely.

A cell-free system for fibroin synthesis has been established by Ejiri[24,25] and Taira[26]. The procedure of preparation of ribosomes from the silk gland differs considerably from those employed for usual tissues such as liver and reticulocytes. Active polysomes of the silk gland sediment at a relatively low centrifugal force (14,000 × g for 30 min). To disperse the resulting massive precipitate, we usually apply 1% deoxycholic acid followed by a brief sonication.

In the polysome system obtained by the method described above, the elongation reaction of the peptide chain takes place most actively in the presence of aminoacyl-tRNAs, GTP and elongation factors. It was demonstrated that 3 elongation factors, designated as EF-1a, EF-1b, and EF-2, were required for the reaction. EF-1a is concerned in the binding reaction of aminoacyl-tRNA to polysomes in the presence of GTP and EF-1b. The EF-1b was presumed to act as a EF-1a-regenerating factor. The third elongation factor, EF-2, is required for the translocation of peptidyl-tRNA from the amino acyl site of ribosome to the peptidyl site. These results indicate that the functions of EF-1a, EF-1b and EF-2 correspond exactly to that of EF-Tu, EF-Ts and EF-G of *E. coli,* respectively. This is the first observation which shows the occurrence of 3 elongation factors in eukaryotic cells. Thus, we can say as a general principle that at least three protein factors are involved in completing the elongation cycle both in prokaryotes and eukaryotes.

3. Genetic aspects of fibroin synthesis

a) Hereditary variants of fibroin

The occurrence of hereditary variants of fibroin of *Bombyx mori,* has been detected recently[6]. As shown in figure 4, fibroin large subunits of a parent strain of silk worm produce a single band on SDS-polyacrylamide gel electrophoresis. From the values of their electrophoretic mobility, fibroin large subunits from various parent strains of *Bombyx mori* were classified into 3 groups, that is, fast(F), moderate(M), and slow(S) moving group[5]. The large subunit of a hybrid strain, on the other hand, exhibited 2 bands having different mobilities which were the same as those of its parent silkworms, respectively. This fact indicates that the alleles which control the fibroin phenotype are codominant.

Using this electrophoretic technique, a linkage analysis of the fibroin gene in the chromosome of *Bombyx mori* was carried out. The fibroin gene was found to be linked with the Nd gene located on the 23rd

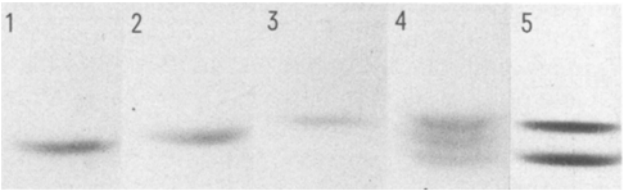
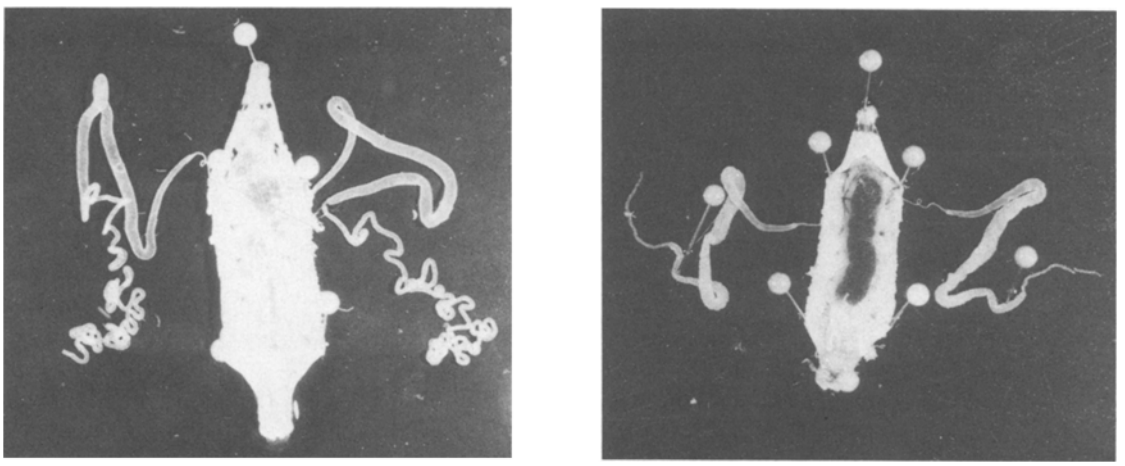


Figure 4. Gel electrophoretic profiles of fibroin large subunits from parent strains of *Bombyx mori* and their hybrid. Parent strains 1, J-131; 2, J-139; 3, J-138; 4, a mixture of J-131, J-139 and J-138; 5, a hybrid of J-131 and J-138.



a) Normal strain. b) Nd(2) strain.

Figure 5. Silk glands at the 5th day of the 5th instar.

chromosome[27]. The posterior silk gland of the Nd silkworm is markedly degenerated, whereas the middle silk gland is almost normal (fig. 5).

The silkworm bearing the Nd mutation is called naked pupa. The Nd mutant produces only a trace amount of fibroin. An attempt to obtain a recombinant between the Nd and fibroin genes has been made. 15 recombinants were obtained in about 300 hybrids (offsprings), suggesting a fairly close distance between the 2 genes. A possibility that the Nd mutation lay on the regulatory region of fibroin gene may be considered, taking into account the evidence that the structure gene of fibroin in the Nd mutant is almost normal[28]. Thus, a comparative study of the fibroin genes of Nd and wild strains of the silkworm will provide a promising system for the study of the regulation of specific gene expression.

b) Fibroin mRNA

The large subunit of fibroin of *Bombyx mori* has a very simple amino acid composition and sequence. About 85% of the amino acid residues of fibroin are glycine, alanine and serine, with glycine comprising 49% of the total and alterating with other amino acids throughout most of the molecule. Assignment of codons for these amino acids predicts that the fibroin mRNA should have a minimum G+C content of 57% with a G content of about 40% or more of the total base residues[5]. In addition, a large value of fibroin's mol.wt predicts an mRNA size of about 5.1×10^6 daltons. Taking advantage of these expected unique features of fibroin mRNA, Suzuki and Brown[29] isolated and purified the fibroin mRNA from the posterior silkgland. The G+C content of a purified fibroin mRNA preparation was 63%, indicating its purity of about 90%.

The average mol.wt of the fibroin mRNA was estimated at 5.6×10^6 daltons (1.6×10^4 base residues) by electron microscopic observation and electrophoretic mobility[30]. This value would be expected to code for a protein of 414,000 daltons, a value about 12% larger than the mol.wt for fibroin itself.

Quantitative measurements of fibroin mRNA in the posterior silk glands was performed by Suzuki and Suzuki[31]. As shown in table 2, a small amount of fibroin mRNA was detected even at the feeding stage on the 3rd and 4th instars. At the molting stage, however, fibroin mRNA disappeared rapidly. At the 5th instar, the synthesis of fibroin mRNA starts again and continues up to at least the 6th day, when fibroin mRNA comprises about 3.5% of the cellular RNA weight. Such a marked accumulation of fibroin mRNA may be partly attributable to a high stability of the fibroin mRNA in the silk gland cell at the 5th instar. At present, no observation suggesting specific amplification of fibroin gene has been made.

As mentioned above, Ohmachi and Shimura[21] have demonstrated that the poly A containing RNA fraction prepared from the posterior silk gland gave an additional RNA fraction, which was smaller than the fibroin mRNA reported by Suzuki et al.[29]. The new RNA was proposed to be the messenger RNA coding for fibroin small subunit by analyzing its translation product by gel electrophoresis and immunological method using an antiserum against the fibroin small subunit. Garel et al.[32] also have presented evidence to indicate that the poly A containing RNA isolated from the posterior silk gland serves really as a mRNA for a small-size component of silk proteins (they call this protein P25 protein).

Thus, we can conclude that 2 major mRNAs, large and small in size, are synthesized in the posterior silk gland at the 5th instar and used effectively as templates for 2 kinds of silk proteins, that is, large and small components of silk proteins, respectively. Although the physiological function of the small component protein is still unknown, it may be of considerable interest to inquire into whether this protein is concerned with some processes of secretion and spinning of silk proteins.

Table 2. Accumulation of fibroin mRNA in the posterior silk gland of *Bombyx mori* through the 3rd to 5th instar**

Stages	Gland weight*	Bulk RNA*	Fibroin mRNA weight*	
	mg	μg	μg	% of total
3rd instar				
Feeding stage	1.1	9	0.21	2.1
Molting stage	1.6–9	13	<0.05	<0.4
4th instar				
Feeding stage	9.2	100	2.1	2.1
Molting stage	11	120	<0.6	<0.5
5th instar				
4th ecdysis	18	250	-	-
1 day	40	500	<2	<0.5
2 days	100	2000	20	1.0
3 days	250	4500	90	2.0
6 days	320	4900	170	3.5

*Amount in a pair of posterior silk glands. **Data from Suzuki and Suzuki[27].

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V. Endocrinological aspects of silk production

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It is usual to associate gland secretion of given protein(s) with the stimulation of a specific hormonal effector. This general picture is valid from some well known tissues such as the oviduct which produces ovalbumin and related white egg proteins under the action of progesterone, and the hen liver which secretes the yolk protein, phosvitin, under oestradiol action. Prolactin induces various lactalbumines in the mammary gland. In avians and mammals, this mode of action as well documented: nuclear receptors have been identified, kinetics investigated and antagonists studied. Nevertheless, the final molecular basis for the hormonal activity remains obscure.

What is the effect of hormonal stimulation on silk proteins' secretion by the silk gland cells in *Bombyx mori?* The key role of ecdysteroids and juvenile hormones in the general development and activity of the silk gland is obvious as is the correlation of its activity and the molting cycle. Despite their interest, endocrinological aspects of silk production are very little investigated and documented. A main question can be raised: are ecdysones and/or juvenile hormones really involved in silk secretion? Is there a specific 'silk secretion factor' responsible for the repeated turning on and off of fibroin and sericin genes?

Quantitative assays of the fibroin mRNA[1] showed that the cognate gene transcription is turned off during the larval apolysis after which the pre-existing fibroin mRNA molecules are completely degraded. Fibroin gene transcription turns on after re-feeding which follows ecdysis. A similar behavior is also true for the P25 mRNA, coding for a small silk protein[2].

1. Ecdysteroids

Since the pioneer observations of S. Fukuda in 1940, who demonstrated by ligation and transplantation experiments that prothoracic glands release active substance(s) in the silkworm blood at critical periods, we know that larval and pupal molts are controlled by ecdysteroid hormones. They were purified for the first time by Butenandt and Karlson in 1954 using silkworm male pupae, formerly described as the α-ecdysone in 1965. A 2nd molting hormone, the β-ecdysone or ecdysterone (20-hydroxy ecdysone) was also isolated from pupae by Hoffmeister in 1966. Both are C-27 sterols with pronounced polar properties.

a) Quantitative assays

Several attempts at quantitative assays based either on bioassays using a *Calliphora* test or more recently radioimmunoassays (RIA) combined with further chromatographic fractionations[3] yield accurate data on free and conjugate ecdysteroids all along the larval cycle of *B. mori* for many tissues (ovaries, pupae, embryos)[4-8]; but surprisingly not yet for silk glands.

Ecdysone changes during larval and pupal development have been described by Calvez et al.[9] in the hemolymph of *B. mori* and *Philosamia cynthia,* of the tobacco hornworm *Manduca sexta* by Gilbert's group[10,11] and recently for *Galleria mellonella* by