Biochemical Genetics, Vol. 20, Nos. 1/2, 1982

Genetic Variants of the *Bombyx mori* Silkworm Encoding Sericin Proteins of Different Lengths

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Received 3 Apr. 1981—Final 22 July 1981

A variant sericin polypeptide originally found by acid gel electrophoresis in the Nd-s *mutant strain of the silkworm,* Bombyx mori, *has been analyzed genetically. The variant polypeptide (called S-2^v) is encoded by a gene which behaves as a codominant allele of the gene encoding the standard S-2 sericin polypeptide. Linkage analysis locates these alleles at 0.0 map unit on chromosome 11. SDS–polyacrylamide gel electrophoresis shows that the molecular weight of the S-2^v variant polypeptide is lower by approximately 62,500 than that of the S-2 polypeptide. Amino acid analysis indicates that the two sericin polypeptides have similar compositions. These results are consistent with the idea that the variant allele arose by deletion within the S-2 coding sequence in the* Src-2 *gene locus as the result of unequal recombination.*

KEY WORDS: gene locus; molecular weight; polyacrylamide gel electrophoresis; sericin protein; *Bombyx mori.*

INTRODUCTION

The silk gland of the silkworm, *Bombyx mori,* synthesizes two classes of silk proteins, fibroin and sericin. Fibroin protein is composed of two polypeptides, one large (360K) and one small (26K), linked by disulfide bonds (Tashiro *et al.,* 1972; Sasaki and Noda, 1973a, b). In the large subunit, the existence of variant polypeptides encoded by codominant allelic genes has been demonstrated (Sprague, 1975; Hyodo and Shimura, 1980). Sprague *et al.* (1979) showed that these variant fibroin polypeptides are encoded by genes of

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0006-2928/82/0200-0165$03.00/0

different lengths which probably arose through unequal recombination. Hyodo *et al.* (1980) used fibroin polymorphism to map the locus of the large fibroin protein, showing that it is linked to the *Nd* (naked pupa) locus on chromosome 23.

Sericin is composed of five major polypeptides synthesized in different sections of the middle silk gland (Gamo *et al.*, 1977). The genetic loci encoding these polypeptides have not been identified, however. The existence of variant sericin polypeptides in certain silkworm strains such as the *Nd* and the *Nd-s* mutants (Gamo, 1973) allows mapping of the corresponding genes. In the present investigation, genetic analysis of these polypeptides has been used to map one of the sericin genes. Further, the molecular weights and amino acid compositions of variant and standard sericin proteins have been compared in order to evaluate possible mechanisms for the production of the variant allele.

MATERIALS AND METHODS

Experimental Animals

A mutant strain (*Nd-s*) derived for the local *Bombyx* variety in Burma was the original source of the variant sericin protein ($S\text{-}2^{v}$). This strain produces abnormal fibroin, as well as variant sericin. A strain producing the $S\text{-}2^{v}$ sericin variant and normal fibroin protein was derived by crossing *Nd-s* and J-124 strains and was used in some experiments. For linkage analysis of the variant sericin gene, stocks of silkworms possessing genetic markers on each chromosome were used. All the larvae used in the present investigation were reared on mulberry leaves at 25–28°C by the usual method (Yokoyama, 1963).

Genetic Analysis

Genetic analysis of variant sericin proteins was carried out by resolving these polypeptides by polyacrylamide gel electrophoresis. Sericin proteins were extracted from spun cocoons or from liquid silk isolated before spinning from the lumen of silk glands. Individuals of the parental, F_1, and F_2 types, as well as those obtained by backcrossing F_1 individuals to parental types, were analyzed with respect to patterns of sericin proteins and inheritance of genetic markers on various chromosomes.

Preparation of Sericin Solutions

Silk proteins, fibroin and sericin, were extracted from the lumen of the middle silk gland with 0.35 M Tris–HCl (*p*H 8.6) containing 8 M urea according to

Gamo *et al.* (1977). Sericin proteins were also extracted from cocoon thread by incubation at 40°C for 2 hr with 0.35 M Tris–HCl (*p*H 8.6) containing 8 M urea and 0.5 M 2-mercaptoethanol.

Acid Gel Electrophoresis

Electrophoresis of silk proteins at acid *p*H was carried out on 5% polyacrylamide gels at *p*H 2.9 containing 4 M urea according to the method of Jordan and Raymond (1969). For each cylindrical gel, approximately 50 μl of proteins was loaded. Gels were stained with 0.1% Coomassie brilliant blue (G-250) (w/v) in a mixture of methanol, acetic acid, and water (5:1:4) at 50°C for 30 min. Acrylamide and bisacrylamide of special grade were purchased from Nakarai Chemical Co., Kyoto.

Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate

SDS–polyacrylamide gel electrophoresis was performed according to Weber and Osborn (1969). Gels were formed in tubes (12.5 cm × 4-mm i.d.). Proteins extracted from the lumen of silk gland or cocoon thread were treated with 10% SDS by boiling for 10 min according to Sprague (1975) and then layered on the gels (4–5% polyacrylamide) in a volume of 10 to 20 μl (approximately 50 μg protein). SDS of special grade (Nakarai Chemical Co., Kyoto) was used without recrystallization.

Estimation of molecular weight by SDS gel electrophoresis was performed by referring to the relative mobility of marker proteins (BDH Chemicals Ltd., Poole, England) composed of monomer (53,000), dimer (106,000), trimer (159,000), tetramer (212,000), pentamer (265,000), and hexamer (318,000).

Two-Dimensional Gel Electrophoresis

The first dimension was acid gel electrophoresis in a vertical slab. The gel was dialyzed for several hours against phosphate buffer (*p*H 7.2) containing 10% SDS, and the second electrophoresis was performed in a horizontal slab gel containing SDS in the buffer described by Weber and Osborn (1969).

Amino Acid Analysis

Sericins S-2 and S-2^{v} were isolated from cocoon thread of the two homozygous strains by salting out (50–60% saturation) with ammonium sulfate in 5 M urea, followed by gel filtration on CPG-10 in the presence of 5 M urea. Isolated proteins were hydrolyzed with 6 N HCl at 110°C for 20 hr. Amino acids were analyzed with an automatic analyzer (JEOL, JLC-6AH).

Sericin Content in Cocoon Shell

The relative amounts of each sericin protein in cocoon thread were determined by densitometric scanning of gels using a dual-wavelength tlc scanner (CS-910, Shimazu Seisakusho Ltd., Kyoto) at 590 nm (λ_S) and 420 nm (λ_R) at scanning and recording chart speeds of 20 mm/min. Amounts of total sericin in cocoon shells were determined by gravimetric methods after the extraction of sericin by boiling the cocoon shell with aqueous solutions of 0.5% Marseilles soap and of 0.1% Na_2CO_3 for 40 min each according to the manual of the Sericultural Experiment Station.

RESULTS

Genetic Analysis of a Variant Sericin

Silk proteins extracted from the lumen of silk glands in the *Nd-s* strain contain sericin S-2^v, which migrates faster than the major polypeptides in

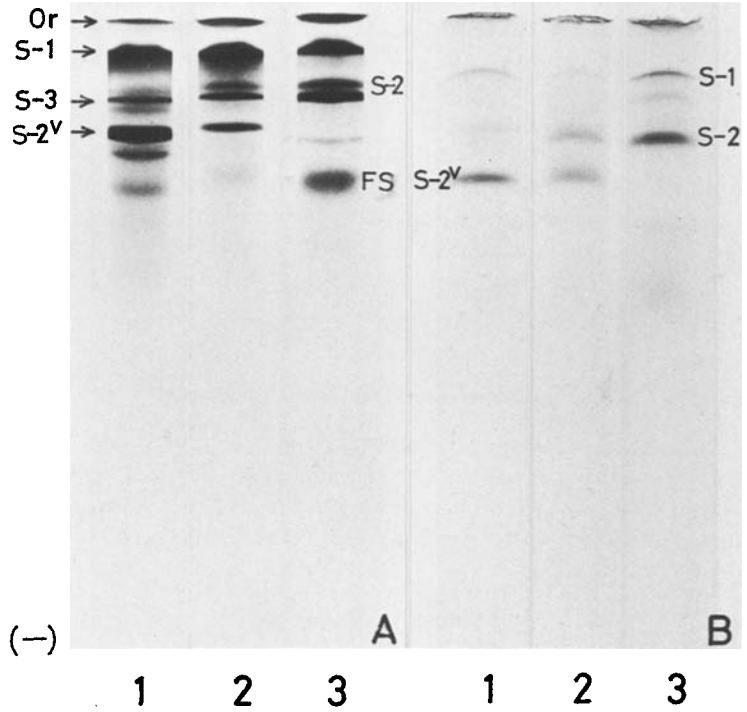

Fig. 1. Electrophoretic patterns of polypeptides in sericin proteins isolated from the silk gland (A) and cocoon thread (B) in F_1 and their parental larvae. Electrophoresis was performed at *p*H 2.9 with 4 M urea. (1) S-2^v/S-2^v; (2) S-2^v/+; (3) +/+.

Table I. Phenotypes of Sericin Proteins in the Offspring Obtained by Various Crosses in the Silkworm

Cross	Phenotype			Total	χ^2 test
	S-2^v/S-2^v	S-2^v/+	+/+[a]		
S-2^v/S-2^v × S-2^v/S-2^v	26	0	0	26	—
+/+ × +/+	0	0	26	26	—
S-2^v/S-2^v × +/+	0	26	0	26	—
S-2^v/+ × S-2^v/+	7	13	6	26	$P > 0.95$
S-2^v/+ × +/+	0	14	12	26	$P > 0.5$

[a]S-2 phenotype was described as +.

normal and *Nd* mutant strains (Gamo, 1973). *Nd-s* strains lack the S-2 sericin protein synthesized by normal silkworms (Fig. 1).

Results obtained by crossing individuals with normal (S-2) and variant (S-2^v) sericin proteins are presented in Table I. The silk proteins extracted from the silk glands of the F_1 progeny from such a cross (Nd-s × normal) are shown in Fig. 1A. The same patterns are seen in the proteins extracted from cocoon thread (Fig. 1B). This result strongly suggests that the synthesis of the S-2^v polypeptide is controlled by a codominant gene allelic to the gene encoding the S-2 polypeptide.

Densitometric analysis of stained gels showed that the amount of S-2^v polypeptide in heterozygous larvae was half that in homozygous animals (Table II). The amount of total sericin proteins in the cocoons produced by S-2^v homozygous larvae was greater than in cocoons from heterozygous and normal larvae (Table II). The discrepancy in total sericin proteins appears to reflect the large amount of S-2^v polypeptide produced by homozygous larvae.

Table II. Peak Area of Three Polypeptides in Sericin Proteins Obtained by Densitometric Scanning of Gels and Percentage of Sericin Proteins in the Thread of Cocoon Shell

	Genotype		
	+/+	+/*Src-2^v*	*Src-2^v*/*Src-2^v*
Peak area			
S-2, S-3	5.11 (26.7)[a]	3.57 (12.7)	1.85 (6.8)
S-2^v	0.00 (0.0)	3.28 (11.6)	6.26 (23.2)
Total	5.11 (26.7)	6.85 (24.3)	8.11 (30.0)
Total sericin (%)[b]	21.5	21.8	23.4

[a]Values in parentheses indicate the proportion of each fraction to the total peak area of sericin proteins.
[b]Percentages of total sericin proteins in cocoon thread.

In the F_2 generation, three phenotypes, S-2^v/S-2^v, S-2^v/+, and +/+, appeared at the expected Mendelian ratio of 1:2:1 (Table I). The appearance of the S-2^v polypeptide in the F_2 generation was not influenced by the segregation of the *Nd-s* phenotype (Fig. 2). Thus, the S-2^v polypeptide is not synthesized by the *Nd-s* gene itself, nor is the gene encoding the S-2^v polypeptide closely linked to the *Nd-s* gene, which is known to be on chromosome 14 (Horiuchi *et al.*, 1963). Further, the appearance of a minor polypeptide (S-X^v) migrating faster than the S-2^v polypeptide was independent of either the S-2^v or the *Nd-s* phenotype.

Backcrosses of normal males (+/+) to F_1 heterozygous females (Nd-s/+, S-2^v/+) yielded two types of progeny, S-2^v/+ and +/+, at the expected ratio of 1:1 in both cases, indicating that the S-2^v and *Nd-s* phenotypes are independent (Fig. 3). Another variant polypeptide (S-X^v) of fast mobility also segregated in the backcross progeny independently of the S-2^v and the *Nd-s* phenotyes.

As the result of these genetic experiments, the gene coding for the S-2^v polypeptide can be considered allelic to the gene coding for the S-2 polypeptide. We have named these genes *Src-*2^v and *Src-2,* respectively.

Map Position of the *Src-2* Gene

Linkage analysis of the major sericin protein variant demonstrated that the *Src-2* gene is linked to the *K* (Knobbed) gene on chromosome 11 as shown in

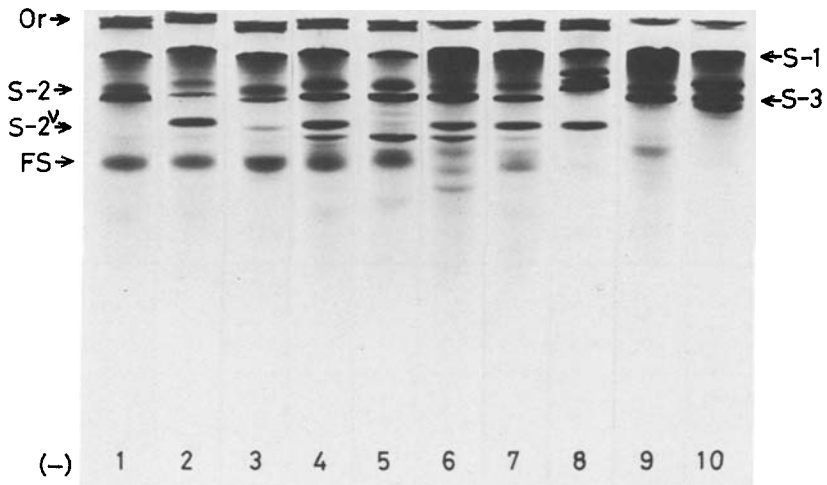

Fig. 2. Electrophoretograms showing the segregation of polypeptides in sericin proteins isolated from the anterior part of the middle silk gland of individual larvae in F_2 progeny obtained by a cross between *Nd-s* and normal moths. The amount of a small fibroin fraction (FS) in *Nd-s* larvae is very small due to the low synthesis of fibroin proteins. (1–5) Normal; (6–10) *Nd-s*.

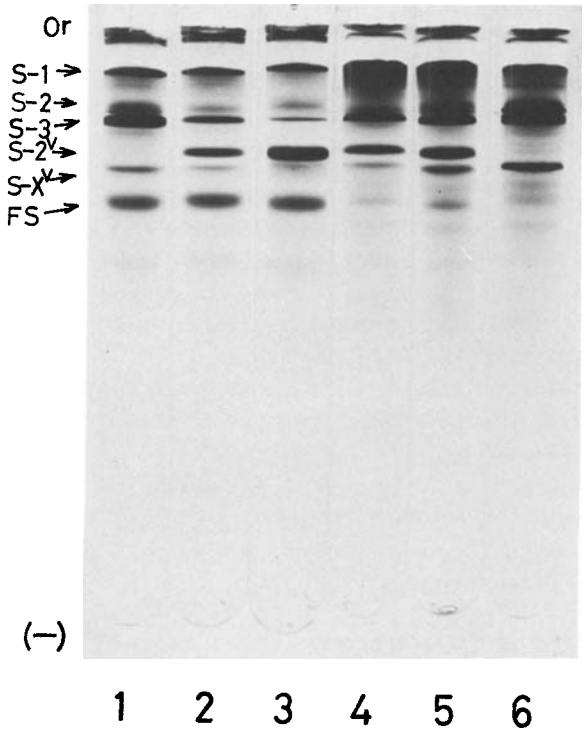

Fig. 3. Electrophoretograms showing the independence of S-2^v, S-X^v polypeptides and the *Nd-s* gene in the BF_1 generation obtained by the cross of Nd-s/+ × +/+.

Table III. A cross between a K +/+ S-2^v female and a ++/++ male yielded no recombinant types, because there is no crossing-over in the female of the silkworm (Tanaka, 1914). However, the progeny obtained from the reciprocal cross between a ++/++ female and a K +/+ S-2^v male included the recombinant types ++ and K S-2^v, and the recombination value between

Table III. Crossing-Over Experiment of the *Src-2^v* with the *K* and *mp* Genes on Chromosome 11[a]

Cross (female × male)	Segregation of characters		Total	Recombination value (%)
	S-2/S-2	S-2/S-2^v		
K +/+ S-2^v × + +/+ +	*K* 5 + 0	*K* 0 + 5	10	—
+ +/+ + × K +/+ S-2^v	*K* 62 + 10	*K* 18 + 60	150	18.67
mp +/mp + × mp +/+ S-2^v	*mp* 65 + 61	*mp* 57 + 97	280	42.14

[a] Independent genes and linkage groups tested: Sex (1), *Ze* (3), *L* (4), *oc* (5), *E* (6), *q* (7), *I-a* (9), *ge* (12), *Nd-s* (14), *bts* (17), *nb* (19), *oh* (20), *rb* (21), and *so* (26).

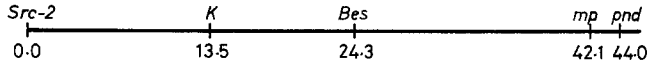

Fig. 4. Location of the *Src-2* gene encoding S-2 and S-2^{v} polypeptides on chromosome 11 of the silkworm.

the *Src-2*^{v} and the *K* genes was calculated at 18.7%. In the analysis of recombination between *Src-2*^{v} and *mp* (micropterous wing), another gene located on the same chromosome, the progeny obtained from a cross between a mp +/mp + female and a mp +/+ S-2^{v} male included recombinant types + + and mp S-2^{v}, and the recombination value between the *Src-2*^{v} and the *mp* genes was calculated at 42.1%.

Since the map distance between *K* and *mp* on chromosome 11 is 28.9 (Yamamoto *et al.*, 1978), the *Src-2* gene must be located to the left of both *K* and *mp*, as shown in Fig. 4. Taking the value of 42.1 for the map distance between *Src-2* and *mp*, *Src-2* is located at position 0.0 with respect to the other chromosome 11 loci shown in Fig. 4.

Molecular Weight

In two-dimensional gel electrophoresis, the S-2^{v} and S-3 polypeptides had the same relative mobilities in the SDS gel as in the acid gel (Fig. 5). Therefore, the faster-moving polypeptide in SDS gels can be identified as S-2^{v}. Repre-

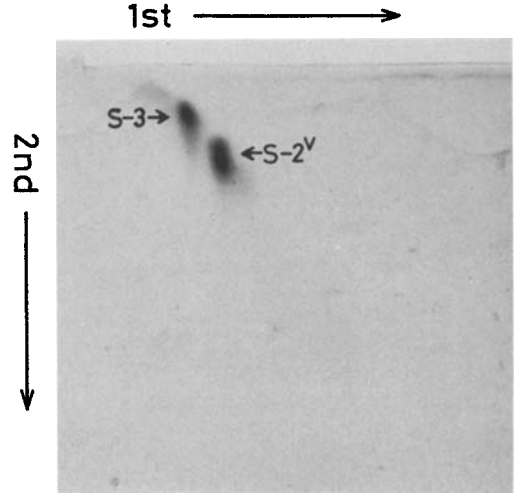

Fig. 5. Two-dimensional gel electrophoresis showing the identification of the S-2^{v} polypeptide in sericin proteins on SDS gels. First electrophoresis, *p*H 2.9 with 4 M urea; second electrophoresis, *p*H 7.2 with SDS.

sentative SDS gel patterns of sericin proteins extracted from cocoon thread produced by homozygous normal and *Src-2*^{v} larvae and heterozygous +/ *Src-2*^{v} larvae are shown in Fig. 6. Molecular weights of three major polypeptides were estimated by referring to the relative mobility of marker proteins (Fig. 7). The S-2 and S-3 polypeptides are similar in molecular weight, 226,500 and 218,800, respectively, while the molecular weight of S-2^{v} is 164,000. The S-2^{v} polypeptide is thus smaller by approximately 62,500 daltons than the S-2 polypeptide.

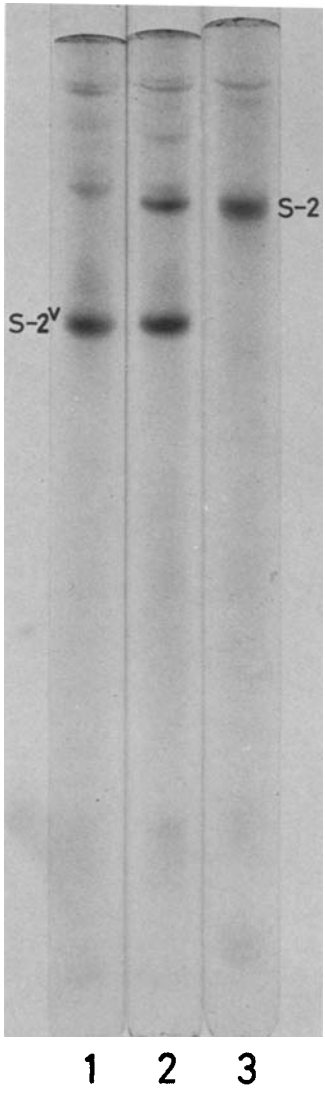

Fig. 6. SDS gel electrophoresis of sericin proteins extracted from cocoon threads produced by individual larvae constituting different genotypes in the *Src-2* allele. (1) *Src-2*^{v}/*Src-2*^{v}; (2) *Src-2*^{v}/+; (3) +/+.

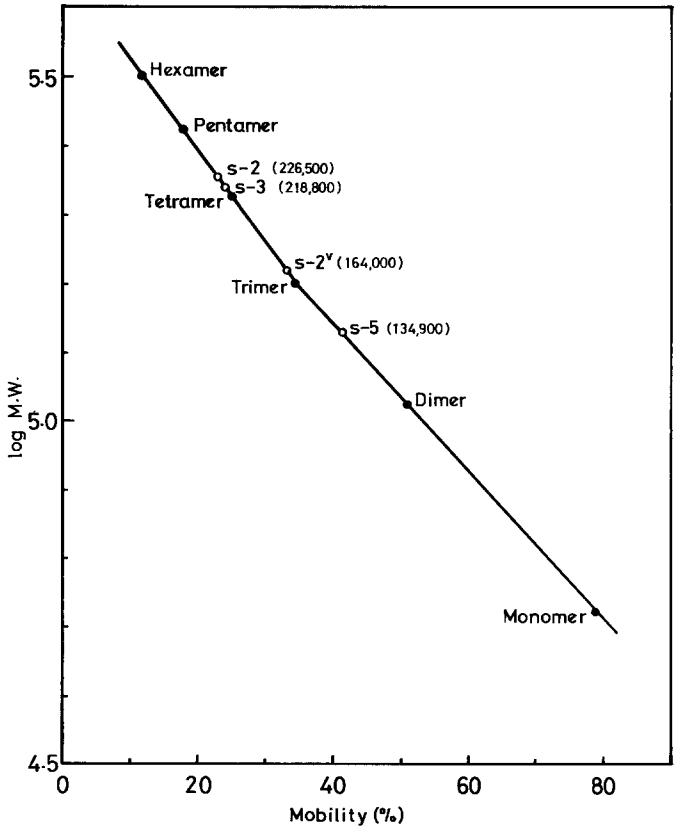

Fig. 7. Estimation of the molecular weight of four polypeptides in sericin proteins by SDS gel electrophoresis on 4% polyacrylamide. See text for molecular weights of marker proteins.

Amino Acid Composition

Two polypeptides, S-2 and S-2^{V}, were isolated from cocoon thread by ammonium sulfate precipitation and gel filtration. The purity of both sericins isolated in this manner was about 95% by densitometric measurement of proteins after fractionation by gel electrophoresis. The results of amino acid analyses performed on S-2 and S-2^{V} polypeptides are shown in Table IV. The amino acid compositions of the two proteins are similar. However, some differences were observed. In particular, the amounts of glycine, leucine, isoleucine, and glutamic acid are higher in S-2^{V} than in S-2. Serine and lysine showed the opposite relationship. However, the discrepancy in amino acid composition between the two polypeptides may reflect contributions from contaminating proteins.

Table IV. Comparative Analysis of Amino Acid Compositions[a] in S-2 and S-2^{v} Polypeptides of Sericin Proteins

Amino acid	S-2	S-2^{v}
Asp	14.9	14.4
Thr	4.0	4.1
Ser	36.8	30.8
Glu	11.1	13.6
Pro	Trace	Trace
Gly	14.9	17.0
Ala	4.4	5.5
Val	1.2	1.8
Cys	0.0	0.0
Met	0.0	0.0
Ileu	0.6	1.3
Leu	1.4	2.6
Tyr	0.1	0.5
Phe	0.4	0.8
Lys	6.0	2.9
His	1.0	0.9
Arg	3.2	3.7

[a]Mean values of each two analyses at 110°C for 20 hr.

DISCUSSION

Sericin synthesized in the normal silk gland of the silkworm is composed of five major and several minor polypeptides, synthesized in different sections of the middle silk gland (Gamo *et al.*, 1977). The existence of two variant polypeptides has been reported in sericin proteins from the *Nd-s* mutant silkworm strain (Gamo, 1973). Genetic analysis of sericin protein variants demonstrates that a major variant polypeptide, S-2^{v}, is encoded by a gene named *Src-2^{v}* which is allelic to the *Src-2* gene encoding the S-2 polypeptide. The *Src-2* locus is at 0.0 map unit on chromosome 11 (Fig. 4).

The molecular weight of the variant S-2^{v} sericin was lower by approximately 62,500 than that of the S-2 polypeptide. Possibly, the *Src-2* allele arose through deletion of nucleotides as a result of unequal recombination. The size of the required deletion in coding region would be about 1.5 kb, assuming the mean molecular weight of amino acids in sericin proteins to be 125. A deletion of this size in sericin genes seems reasonable by comparison with the value of ~1 kb for the difference in length of fibroin genes encoding long and short fibroin proteins in the silkworm (Sprague *et al.*, 1979). Three sericin variants similar to the S-2^{v} polypeptide in electrophoretic mobility were recently found in some local varieties (Gamo, unpublished). Several size variants in the large fibroin protein have also been observed (Lizardi, 1979; Hyodo and Shimura, 1980). Therefore, size variations in sericin proteins

should be considered to occur at relatively high frequencies and variant sericins are not restricted to the mutants of *Nd* and *Nd-s*.

The amount of sericin in the cocoon thread produced by homozygous *Src-2*^{v} larvae is higher than that from normal and heterozygous animals. The amount of S-2^{v} polypeptide in homozygous *Src-2*^{v} larvae is also greater than the amount of S-2 polypeptide in normal larvae. This finding suggests that the *Src-2*^{v} allele might have arisen by tandem duplication of a short variant allele at the *Src-2* locus. Duplication of coding regions is an important process in the evolution of many organisms (Ohno, 1970). In the silkworm, duplication of genes is likely to have occurred in an integument esterase (Eguchi and Yoshitake, 1966), small blood lipoproteins (Gamo, 1978), and chorion proteins in eggs (Regier *et al.*, 1978).

Amino acid analysis showed that the S-2 and S-2^{v} polypeptides are similar in composition. It is not yet clear whether the small discrepancies in amino acid composition we observed are the result of deletions of part of the coding region in the *Src-2* gene. Further study is necessary to elucidate the differences in internal structure of the two alleles. Sprague *et al.* (1979) compared the structures of two alleles in the large subunit of fibroin protein of the silkworm by using restriction endonuclease sites. This technique should also be useful for comparing the structures of sericin alleles, and it will be applicable to sericin genes in the silkworm if their mRNA can be isolated.

It is unlikely that all silk proteins are encoded by closely linked genes. The present investigation shows that a minor variant sericin polypeptide (S-X^{v}) found in *Nd* and *Nd-s* mutant larvae is not linked to the *Src-2* gene. Moreover, the *Fib* gene encoding the large subunit of fibroin protein is located on a different chromosome from *Src-2* (Hyodo *et al.*, 1980). However, some variant polypeptides were found recently in three fractions with different mobilities on SDS gels, and the genes encoding these variant sericin proteins are also linked with the *K* gene on chromosome 11 (Shonozaki *et al.*, 1980; Haga *et al.*, 1980). Therefore, genes encoding the major sericin proteins are considered to be located on a single chromosome. Since different silk proteins are synthesized in different parts of the middle silk gland (Machida, 1927; Gamo *et al.*, 1977), the activity of at least some of the corresponding genes may be controlled independently in particular cell types. We are interested in understanding the organization and structure of these genes in order to examine the mechanisms by which they are regulated.

ACKNOWLEDGMENT

I am grateful to the late Mr. Y. Kobari for his help in determining the sericin content in cocoon shells.

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