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Light-chain fibroin of *Galleria mellonella* L.

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Abstract The posterior section of *Galleria mellonella* silk glands contains two abundant mRNAs that are identical except for the non-coding tail, which includes either two (1.1 kb mRNA) or three (1.2 kb mRNA) consensus sequences for polyadenylation sites. The transcripts are 40% homologous in the coding as well as non-coding regions with the mRNA encoding light-chain fibroin (L-fibroin) in *Bombyx mori*; the deduced translation product shows 43% identity with the *Bombyx* L-fibroin peptide, with all three cysteines conserved. Amino acid analysis of the N-termini of *Galleria* silk proteins revealed that L-fibroin (25 kDa) occurs in two isoforms, the shorter one lacking the Ala-Pro dipeptide residue at its N-terminus. The 29 and 30 kDa *Galleria* silk proteins appear to be homologs of *Bombyx* silk component P25. The results suggest that evolutionary diversification of *Galleria* and *Bombyx* L-fibroins involves alternative polyadenylation and proteolytic processing sites.

Key words Fibroin · DNA sequence · Protein sequence · Alternative polyadenylation · Proteolytic processing

Introduction

The silk glands of certain caterpillars synthesize large amounts of various proteins that are spun out as silk fibre. Proteins produced in the posterior silk gland region form the core fibre of commercial silk. The proteins have been extensively studied in the domestic silkworm, *Bombyx mori*, in which the fibre consists of ca. 350 kDa heavy-chain fibroin (H-fibroin), and several smaller moieties (Sasaki and Noda 1973), including a 25 kDa light-chain fibroin (L-fibroin; Kimura et al. 1985), and another protein of similar deduced size, P25 (Couble et al. 1983). Peptide and nucleic acid analyses (Chevillard et al. 1986, Yamaguchi et al. 1989), have shown that L-fibroin and P25 are unrelated.

From the waxmoth, *Galleria mellonella*, we isolated two partial cDNAs specific for the posterior silk gland (Žurovec et al. 1992). One of the cDNAs hybridized to a ≥10 kb transcript that corresponds to H-fibroin mRNA on the basis of nucleotide sequence, size, and site of synthesis. The other cDNA, designated PG-1, hybridized to two transcripts of 1.1 and 1.2 kb in size . Although sequence analysis of PG-1 suggested that it is homologous to the *Bombyx* L-fibroin transcript, this incomplete cDNA did not cross hybridize with *Bombyx* L-fibroin cDNA.

The posterior silk gland of *Galleria* was previously shown to secrete three proteins of similar size: 24, 29 and 30 kDa (Grzelak et al. 1988). It was suggested that the two mRNAs of 1.1 and 1.2 kb that hybridized to PG-1, encode two of these polypeptides (Žurovec et al. 1992). However, that was found not to be the case; we report here that both PG-1 mRNAs encode a single L-fibroin protein and that the 29 and 30 kDa *Galleria* silk components are homologous to two P25 polypeptides of *Bombyx*.

Materials and methods

Insects, tissues and cocoons

Larvae of the waxmoth, *G. mellonella* L. (Lepidoptera, Pyralidae) came from our standard laboratory culture (Sehnal 1966). Posterior sections of silk glands were dissected from water-anaesthetized larvae under Ephrussi-Beadle saline. They were rapidly frozen in liquid nitrogen or on dry ice and stored at –80°C. A French commercial hybrid of *B. mori* L. (Lepidoptera, Bombycidae) Z20×Z26 was purchased from International Sericultural Commission, Lyon, France, and reared on mulberry leaves in the usual way to obtain cocoons for silk analysis. Cocoons from a Japanese hybrid NO2×CO2 were obtained from Prof. Hiromu Akai, Tokyo Agricultural University.

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Nucleic acid analysis

A 500 bp EcoRI-Sau3A fragment derived from the 5′end of PG-1 cDNA (Žurovec et al. 1992) was radiolabelled with a nick-translation kit (BRL/Gibco) and used as a probe to screen a new cDNA library and for Northern analysis. Northern blots were also analysed with the synthetic oligonucleotide 5′ GTATCGAATAC-CGTGAAATGAAAAGCCAGGTCGTG 3′, which is complementary to nucleotides 1108–1142 of the cDNA fragment (Fig. 1). The oligoprobe was end-labelled with polynucleotide kinase (10 pmol of oligonucleotide labelled with 50 μCi ATP), and purified on a Sephadex G25 column (10×1 cm).

The cDNA library was based on RNA from the posterior silk glands of day-3 last instar larvae. Kits were used to isolate poly(A)$^+$ RNA (Pharmacia-LKB) and to prepare a cDNA library in lambda gt10 (Promega).

The unamplified library (titre of 10^6 pfu) was screened with the 500 bp fragment under conditions identical to those in our previous study (Žurovec et al. 1992). Selected cDNA inserts were subcloned in pGEM 3Z and sequenced with the T7 sequencing kit (Pharmacia-LKB), using T7 and SP6 primers.

RNA for the Northern blot analysis was prepared as described earlier (Žurovec et al. 1992). Blots were hybridized to nick-translated probes under high stringency conditions at 65°C in 6×SSC, 0.5% sodium dodecyl sulphate (SDS); washing at 65°C twice in 2×SSC, 0.2% SDS and twice in 0.2×SSC, 0.1% SDS (1×SSC is 0.15 M NaCl, 15 mM sodium citrate). Hybridization to the end-labelled oligoprobe was carried out at 37°C in 5×SSPE (1×SSPE is 0.18 M NaCl, 10 mM Na_2HPO_4, lmM EDTA pH 7.7), 5×Denhardt's solution, 0.5% SDS; membranes were washed twice in 2×SSPE, 0.1% SDS at room temperature and twice in 1×SSPE, 0.1% SDS at 37°C.

Polypeptide separation and sequencing

Silk proteins were isolated from cocoons according to the following procedures. Clean cocoons were chopped and solubilized for 24 h in 10 mM Tris-HCL buffer (pH 7.0) containing 5% 2-mercaptoethanol, 2% SDS, and 8 M urea. Insoluble H-fibroin was separated by centrifugation (5 min at 60×*g*). The dissolved polypeptides were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in a 20% gel and visualized with silver nitrate (Görg et al. 1985, as modified by Kopáček et al. 1988).

The polypeptides were electroblotted from the gel onto a polyvinylidene difluoride (PVDF) membrane (Schleicher and Schuell) for N-terminal microsequencing according to Matsudaira (1987). The gels were soaked for 5 min in transfer buffer [10 mM 3-(cyclohexylamino)-1-propane-sulphonic acid in 10% methanol, pH 11.0] and subjected to electroblotting to PVDF membrane for 10–30 min at 0.5 A. The membrane with attached peptides was washed in deionized water for 5 min, stained with Coomassie Blue (0.1% in 50% methanol) for 5 min, washed in 50% methanol with 10% acetic acid for 5–10 min, rinsed in deonized water, air dried, and stored at –20°C. Sequencing was performed on an Applied Biosystems 477A protein sequencing apparatus at the Protein/Nucleic Acid Shared Facility, Medical College of Wisconsin, Milwaukee, Wis., USA.

Antibodies and Western blotting

Rabbit antibodies to *Bombyx* L-fibroin and to synthetic fragment of 25 residues from the deduced sequence of P25 (Tanaka et al. 1993) were kindly provided by Prof. S. Mizuno of Tohoku University. Solubilized cocoon proteins were separated by PAGE and electroblotted onto nitrocellulose membranes (Schleicher and Schuell), which were washed with shaking in the following solutions (Johnson et al. 1984): (1) 5% skim milk (Difco), 1 h; (2) 0.02 M phosphate-buffered saline (PBS) containing 0.05% Tween 20, 5×5 min; (3) primary antiserum diluted with PBS Tween (anti-L-fibroin antibody 1:10000 and anti-P25 antibody 1:500), 1 h; (4) PBS Tween, 5×5 min; (5) anti-rabbit porcine IgG conjugated with horseradish peroxidase (Sevac, Praha), diluted 1:1000 with PBS Tween, 1 h; (6) PBS Tween, 5×5 min; (7) 0.01% hydrogen peroxide and 0.025% o-diaminobenzidine in 0.1 M Tris (pH 7.6), 1–5 min; (8) distilled water.

Results

Sequence analysis of L-fibroin cDNA and identification of alternative polyadenylation sites

Previously we reported the sequence of PG-1, a partial cDNA clone from *G. mellonella* with apparent homology to *Bombyx* L-fibroin (Žurovec et al. 1992). Using the 500-bp 5′ fragment of PG-1 as a probe, we have now identified 12 positive clones among about 20000 plaques from the new cDNA library. Their inserts ranged in size from 1000 to 1200 bp.

A composite *Galleria* PG-1 cDNA, of 1182 nucleotides in length containing the entire reading frame, was assembled using the sequence data from the various clones (Fig. 1). The methionine initiation codon, which was identified at position 15, is located in an environment that is optimal for translational initiation (Kozak 1989). The translation termination codon TAA is at +816. The PG-1 nucleotide sequence is 40% identical to that of *Bombyx* L-fibroin cDNA (Fig. 1), and the deduced amino acid sequences show 43% identity and 75% similarity (Fig. 2), thus confirming that PG-1 is a waxmoth homolog of *Bombyx* L-fibroin gene. Southern blot analysis of waxmoth genomic DNA indicated that PG-1 is a single-copy gene (Žurovec et al. 1992), like the L-fibroin gene in *Bombyx* (Kikuchi et al. 1992).

PG-1 cDNA clones fell into two categories that differ in the length of the 3′ non-coding region. For example, clones PG-1/2 and PG-1/6 terminate in long poly(A) tails, indicating that they are derived from functional mRNAs, and have identical sequences except that PG 1/6 is 79 bp longer than PG-1/2 (Fig. 1). PG-1/6 includes three polyadenylation consensus sequences (AATAAA) at positions 978, 1054 and 1154, while the PG-1/2 clone lacks the last polyadenylation signal and its poly(A) tail begins at position 1104. Both PG-1/6 and PG-1/2 clones recognize the two mRNA species of 1.1 and 1.2 kb, but only the 1.2 kb mRNA can be identified with the 3′ end-specific oligoprobe (Fig. 3A). These data demonstrate that the PG-1/6 cDNA is derived from the 1.2 mRNA and the PG-1/2 cDNA from the 1.1 kb mRNA.

Developmental Northern analysis of L-fibroin transcripts

Both the 1.1 and 1.2 kb transcripts occur in the posterior silk glands throughout the penultimate (data not shown) and last larval instars (Fig. 3B). The abundance of the longer transcript declines in immobile prepupae, and both transcripts disappear within the next 12 h when the insects pupate; this occurs about 3 days after the termination of feeding. When feeding is prevented and develop-

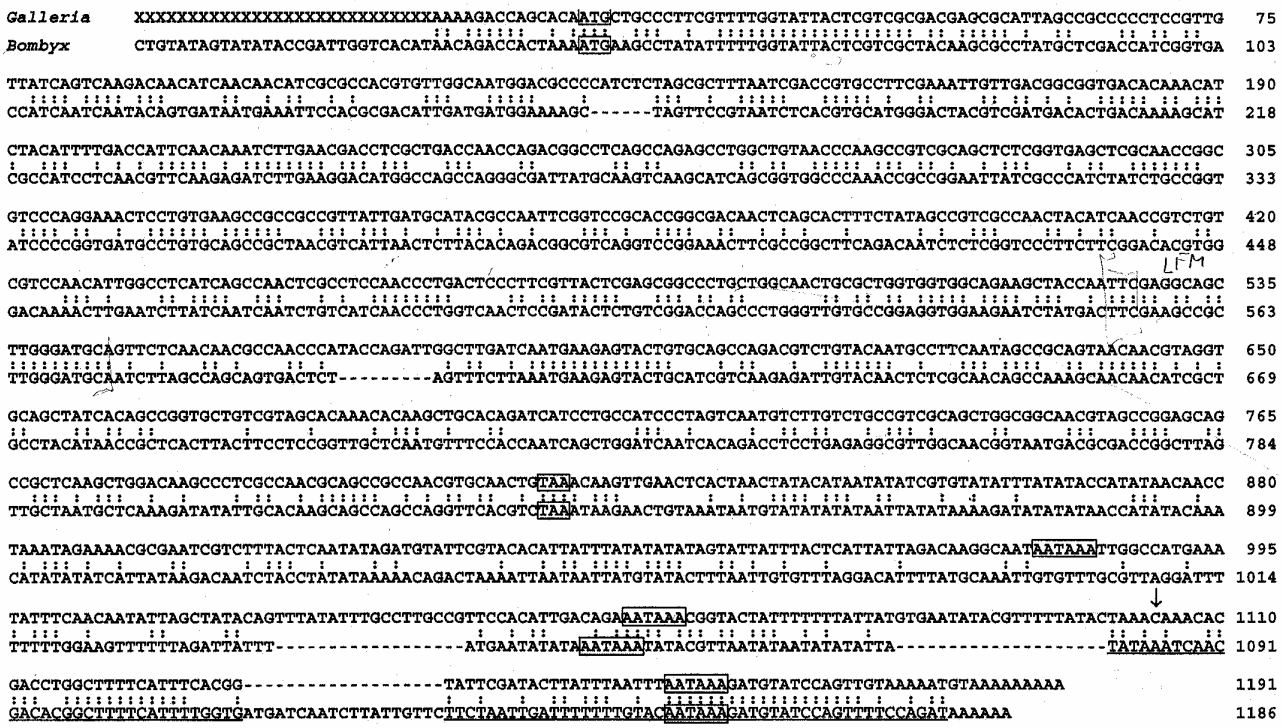

Fig. 1 Nucleotide sequence alignment of full-length *Galleria mellonella* L-fibroin and *Bombyx mori* L-fibroin cDNAs. Matching nucleotides are indicated by *colons*; the ATG initiation codons, TAA termination codons, and polyadenylation motifs AATAAA are *boxed*. The *arrow* indicates the end of the PG-1/2 cDNA clone corresponding to the 1.1 kb *Galleria* L-fibroin mRNA. The full-length cDNA is derived from the 1.2 mRNA; the 3' regions of high homology with *Bombyx* L-fibroin mRNA are *underlined*

ment arrested by ligating 5-day old larvae across the mesothorax, the transcripts remain detectable for at least 5 days (Fig. 3B).

The *Galleria* L-fibroin protein

The open reading frame in the L-fibroin cDNA codes for a polypeptide of 267 amino acid (Fig. 2). Based on estimates of the probable site of signal peptidase activity (von Heijne 1986), the deduced polypeptide includes a 16-amino acid signal peptide and thus the mature L-fibroin protein would contain 251 amino acid residues with a M_r of 25,442. The size corresponds to the *Galleria* cocoon component that appears on the electrophoretograms as a single band of 25 kDa (Fig. 4). Sequencing of the material localized in this band revealed that it consists of two polypeptides that differ slightly in N-terminal sequence (Table 1). The first eleven amino acids of the dominant peptide (comprising 73% of the isolated material) match amino acid residues 17 to 27 of the deduced PG-1 polypeptide (Fig. 2). The less abundant second peptide is two amino acids shorter, starting with residue 19. These data, confirmed by sequencing another preparation of the 25 kDa protein, suggest that both peptides represent genuine silk gland products that are isoforms of *Galleria* L-fibroin. The N-terminal sequence of the truncated polypeptide is identical with that of the only known form of *Bombyx* L-fibroin (Table 1).

L-fibroin and other small peptides from posterior silk gland of *Bombyx* and *Galleria*

Electrophoretic analysis of cocoon extracts reveals a variety of protein bands (Fig. 4). It should be noted that the protein compositions of the two analysed *Bombyx* stocks are similar and clearly differ from the protein profile of the *Galleria* cocoon extract, even though some of the bands identified in *Bombyx* have counterparts of similar size in *Galleria*. The most obvious bands of 25 kDa, 27 kDa (*Bombyx* only), 29 kDa (*Galleria* only) and 30 kDa were examined further by N-terminal sequencing and immunological analysis.

As noted above, the 25 kDa component of *Galleria* silk contains two isoforms homologous to *Bombyx* L-fibroin. Western blot analysis revealed that the 25 kDa band from *Bombyx* also includes L-fibroin (Fig. 5). The N-terminal sequences of the 29 and 30 kDa *Galleria* silk peptides are identical, and comparison with known sequences of *Bombyx* silk peptides suggests that these proteins represent *Galleria* homologs of P25 (Table 1). The antibody to *Bombyx* P25 silk peptide recognizes in both analysed *Bombyx* hybrids two protein bands of 27 and 30 kDa. Hence, there are most probably two distinct

Fig. 2 Deduced amino acid sequence of *Galleria* L-fibroin showing homology with *Bombyx* L-fibroin. Identical amino acids are marked with *asterisks*, semi-conservative replacements with *dots*, and Cys residues with *arrows*. *Lines* above and below a stretch of amino acid residues represent the two N-termini established by L-fibroin peptide sequencing

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Galleria  MLPFVLVLLVATSALAAPSVVISQDNINNIAPRVGNGRPISSALID  45
Bombyx    MKPIFLVLLVATSAYAAPSVTINQYSDNEIPRDIDDGKA--SSVIS  43
          * *. ********* ***** *.* . *.*.  ...*..  *..*

RAFEIVDGGDTNIYILTIQQILNDLADQPDGLSQSLAVTQAVAALGELATGVPGNS 100
RAWDYVDDTDKSIAILNVQEILKDMASQGDYASQASAVAQTAGIIAHLSAGIPGDA  98
** . **. * .* ** .*.**.*.* * *  **. **.*. . ...*..*.**..
↓
CEAAAVIDAYANSVRTGDNSALSIAVANYINRLSSNIGLISQLASNPDSLRYSSGP 155
CAAANVINSYTDGVRSGNFAGFRQSLGPFFGHVGQNLNLINQLVINPGQLRYSVGP 153
* ** **..*...**.*. ...  ... .. ... *. **.**  **. **** **
  ↓                                   ↓
AGNCAGGGRSYQFEAAWDAVLNNANPYQIGLINEEYCAARRLYNAFNSRSNNVGAA 210
ALGCAGGGRIYDFEAAWDAILASSDS---SFLNEEYCIVKRLYNSRNSQSNNIAAY 205
*  ****** *.*******.* ....   ...*****  .****. **.***..*

ITAGAVVAQTQAAQIILPSLVNVLSAVAAGGNVAGAAAQAGQALANAAANVQL    267
ITAHLLPPVAQVFHQSAGSITDLLRGVGNGNDATGLVANAQRYIAQAASQVHV    262
***  . . .*  .    *. ..* .*. * . .*  *.* . .*.**..*..
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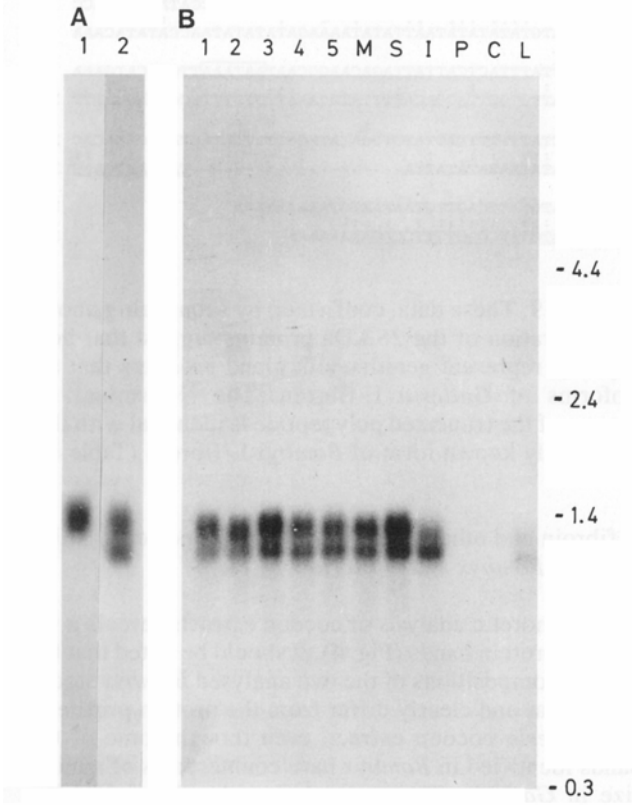

Fig. 3A, B Northern analysis of *Galleria* L-fibroin mRNAs. **A** RNA from posterior silk glands of fully grown larvae was hybridized with the synthetic oligoprobe (lane 1) or with the 5' *Eco*RI-Sau3A fragment (lane 2). **B** Hybridization of the 5' *Eco*RI-Sau3A fragment to posterior silk gland RNA from larvae of different age (lanes 1–5, days after the last ecdysis; lane M, mobile prepupae spinning cocoons; lane S, slowly mobile prepupae; lane I, immobile prepupae within 6 h before pupal ecdysis), from pupae (lane P, about 6 h after ecdysis); lane L, on day 5 after the last ecdysis, larvae were ligated and silk glands were dissected 5 days later. No signal was obtained with RNA from the larval carcass without silk glands (lane C). Sizes are given on the right (in Kb)

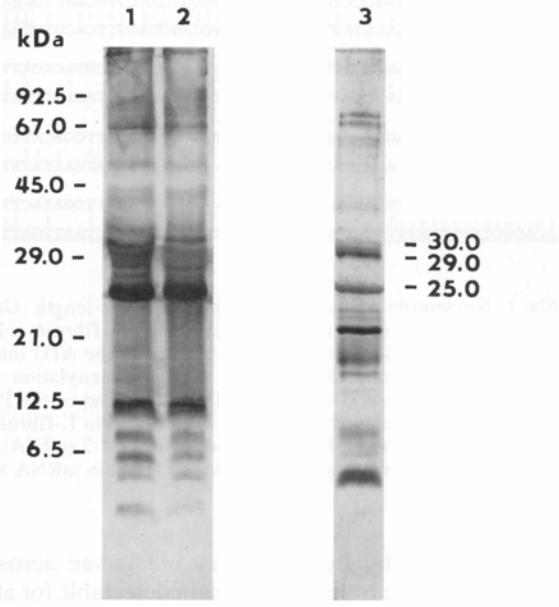

Fig. 4 Electrophoretograms of cocoon polypeptides solubilized in 8 M urea, 2% sodium dodecyl sulphate (SDS) and 5% 2-mercaptoethanol, separated in 20% acrylamide and stained with silver nitrate. Molecular size ladder was established with Protein Test Mixtures 4 and 5 (Serva). Apparent size of analysed bands are indicated in kDa. Lane 1, *Bombyx* hybrid Z20×Z26; lane 2, *Bombyx* hybrid NO2×CO2; lane 3, *Galleria* laboratory stock

Table 1 N-terminal amino acid sequences of *Galleria* silk peptides homologous to *Bombyx* L-fibroin and P25

Galleria 24 kDa protein band	APSVVISEDNI
	SVVISEDNI
Bombyx L-fibroin	SVTINQYSN
Galleria 29 kDa protein band	GPANNVVRPPR
Galleria 30 kDa protein band	xPANNVVRPPRLDDD
Bombyx P25	GPPSPIYRPCYLDDY

[1] The sequenced *Galleria* polypeptides were isolated from cocoons. Data for *Bombyx* P25 were taken from Chevillard et al. (1986) and for *Bombyx* L-fibroin from Yamaguchi et al. (1989)

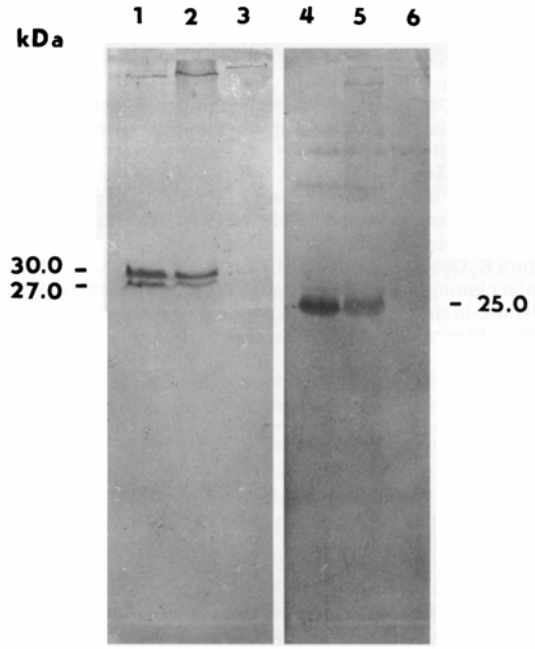

Fig. 5 Reaction of silk peptides with antibodies to *Bombyx* P25 fragment (lanes 1,2,3) and L-fibroin (lanes 4,5,6). Lanes 1 and 4, *Bombyx* Z20×Z26 hybrid; lanes 2 and 5, *Bombyx* NO2×CO2 hybrid; lanes 3 and 6, *Galleria* laboratory stock

size forms of P25 in both *Galleria* and *Bombyx*. Despite the apparent sequence homology between the analysed *Bombyx* and *Galleria* silk peptides, our immunological analysis did not reveal any antigenic similarities: neither the antibody to *Bombyx* P25, nor that to *Bombyx* L-fibroin react with the silk proteins of *Galleria* (Fig. 5).

Discussion

Posterior silk gland polypeptides

The secretion from the posterior silk gland of *Bombyx* contains the large H-fibroin and several small proteins (Sasaki and Noda 1973). The predominating 25 kDa component that dominates the lower molecular weight fraction was named L-fibroin and has been shown to comprise 244 amino acids (M_r of 25800) with an N-acetylated terminal Ser residue (Yamaguchi et al. 1989). By contrast, analysis of silk gland-specific cDNAs from an European silkworm strain led to the conclusion that the 25 kDa silk component is encoded by a gene called P25 (Couble et al. 1983). From the cDNA sequence, the P25 peptide was deduced to comprise 203 amino acids (Chevillard et al. 1986) corresponding to a size of 23778, but the protein has not been isolated. The controversy about the identity of the 25 kDa protein band was resolved with the aid of immunoanalysis by Tanaka et al. (1993). We confirm here that the 25 kDa component of *Bombyx* silk is actually L-fibroin, whereas the 27 and 30 kDa components are derived from the P25 gene (Fig. 5).

In *Galleria*, Grzelak et al. (1988) described three polypeptides of 30, 29, and 24 kDa, as dominant small-sized products of the posterior silk gland. Kodrík (1992) showed that an antiserum to *Galleria* cocoon extract does not recognize any of the *Bombyx* silk proteins, and we show here that none of the *Galleria* silk components reacts with antibodies to the P25 and L-fibroin of *Bombyx*. However, from the data on N-terminal amino acid sequences, and in case of L-fibroin also with the nucleotide sequence, we demonstrate that the 29 and 30 kDa *Galleria* peptides are most probably homologs of the *Bombyx* 27 and 30 kDa products of the P25 gene, while the 25 kDa *Galleria* protein comprises two peptide isoforms homologous to *Bombyx* 25 kDa L-fibroin.

Homology of *Bombyx* and *Galleria* L-fibroins

The degree of homology between the two mRNAs of *Galleria* L-fibroin and the *Bombyx* L-fibroin mRNA (Yamaguchi et al. 1989) amounts to about 73% in the 5′ untranslated and signal peptide coding region (nucleotides 1 to 68) and to 42% in the region encoding the secreted peptide. The longer (1182 nucleotides) *Galleria* mRNA further shares with *Bombyx* L-fibroin mRNA (1180 nucleotides) two highly conserved regions (67 and 76% sequence identity in stretches of 33 and 38 nucleotides, respectively) in the distal 3′ domain (Fig. 1). The 3′ domain of mRNAs is believed to play a role in RNA transport and stability (Yaffe et al. 1985), and the production of mRNA isoforms is often developmentally regulated, as, for example, is the case with murine dihydrofolate reductase (Kaufman and Sharp 1983). However, the short and long isoforms of *Galleria* L-fibroin mRNAs occur concurrently and both persist during larval molting (Fig. 3B), when the *Bombyx* L-fibroin mRNA level declines (Kimura et al. 1985).

The deduced L-fibroins of the waxmoth (267 amino acid residues) and the silkworm (262 residues) exhibit 43.1% identity, but if conservative replacements are included the homology amounts to 74.9%. The most conserved regions are found in the signal peptide and around the Cys residues of the secreted peptide. These data indicate that these regions have been of significance for silk fibre formation for at least 100 million years when the Pyraloidea, which include *Galleria*, and the Bombycoidea, to which *Bombyx* belongs, diverged (Common 1990). Yamaguchi et al. (1989) proposed that two cysteine residues of *Bombyx* L-fibroin form an intramolecular disulphide bridge while the third one links L-fibroin to a Cys residue of H-fibroin. This linkage is reportedly essential for fibroin secretion into the silk gland lumen (Takei et al. 1987).

Post-translational processing of L-fibroin

The studies on *Bombyx* indicated that the signal peptide of L-fibroin is cleaved between Pro and Ser residues at amino acid positions 18 and 19 (Yamaguchi et al. 1989). However, according to von Heijne et al. (1986), Pro is ordinarily not found among the last three residues of signal peptides or as the first residue of the mature peptide, while signal peptide cleavage between two adjacent Ala residues in positions 16 and 17 of *Galleria* and *Bombyx* L-fibroins should occur with 70 to 80% probability. Since the longer isoform of *Galleria* L-fibroin clearly results from this cleavage, we propose that the shorter isoform is generated by further proteolytic processing by which the Ala-Pro (16–17) dipeptide is removed. Various proproteins are processed by removal of the N-terminal dipeptide by type IV dipeptidyl aminopeptidase IV (Kreil et al. 1980). This enzyme has been identified in a lepidopteran insect and shown to possess high specificity for the Ala-Pro N-terminal substrates (Boman et al. 1989).

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