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Purification and Characterization of Glue Proteins Secreted from the Colleterial Glands of the Silkworm, *Bombyx mori*

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ABSTRACT

The glue proteins from the colleterial glands of the adult female silkworm, *Bombyx mori* were purified and characterized by ion exchange chromatography on CM-Sephalose CL-6B, gel permeation chromatography on Sephacryl-400 and electrophoresis. The purified glue proteins showed two major proteins, glue protein-1 (GP-1) and glue protein-2 (GP-2). The molecular weights as determined by SDS-polyacrylamide gel electrophoresis on 7.5% gel were 240 and 190 KDa for GP-1 and GP-2, respectively, while by Sephacryl-400 gel permeation chromatography were 7,000 and 1,700 KDa, respectively. Furthermore, by using SDS-polyacrylamide gel electrphoresis on 15% gel small peptide (SP) was detected with molecular weight of 10 KDa and 26 KDa by Sephacryl-400 column. Amino acid analysis of glue proteins were found to be in unusually high contents of glutamic acid and glycine. NH2-terminal sequences of glue proteins are also reported. Immunological analysis indicates GP-1 is the dominant of glue proteins.

Key words : *Bombyx mori*, colleterial glands, glue proteins

INTRODUCTION

Many insects have evolved the elaborate adhesive mechanisms that allow their eggs to attach to a wide variety of hard surface. The substances mediation adhesion are produced by a female genitarial organ called the colleterial gland or female accessory gland. The colleterial glands have been described occasionally in lepidopterans, but their structure and functions in orthopterans have been extensively studied at the molecular level (Koeppe *et al.*, 1985). The colleterial gland of the silkworm, *Bombyx mori*, is a paired organ composed of common duct (storage lobe) and a secretory lobe with multibranched tubules. The colleterial gland is formed from an imaginal disc located under the 9th abdominal segment and developed suddenly in the last phase of pupal-adult development. At the time of adult emergence large amounts of milky white secretion are depo-

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sited in the storage lobe, and the stored secretion is discharged during egg oviposition (Arisawa and Fugo, 1990; Amornsak *et al* ., 1992). Thus the secretory product is conceived to be used for adhesion of eggs to the substrate where eggs were laid. Proteins are the major components of the secretion and consisted of two major proteins which were separated by a polyacrylamide gel electrophoresis (Amornsak *et al.*,1992). These proteins seem to be the chemical entities responsible for the adhesion of eggs, but no information is available on their properties and structure. The present paper deals with the purification and characterization of glue proteins secreted by the colleterial glands of silkworms.

MATERIALS AND METHODS

Biological materials

The hybrid races (Shunrei x Shogetsu and Kinshu x Showa) of the silkworm, *Bombyx mori*, were used. The larvae were reared on fresh mulberry leaves or alternated with artificial diet (Vitasilk, Kyodo-Shiryo Co., Yokohama) at 25°C to 27°C under 12L : 12 D photoregime. The newly emerged female adults were dissected and the colleterial glands were collected in a saline solution (0.75% NaCl). After rinsing with saline, the colleterial glands were cut at the posterior end and thoroughly squeezed by a fine forceps to collect secretory products. The secretions were briefly centrifuged to remove broken tissue fragments and the resulting supernatant fraction was dialyzed against 50 mM phosphate buffer, pH 7.0, with three changes overnight and used as the crude sample for purification.

CM-Sephalose CL-6B chromatography

All procedures for purification were conducted at 4°C or in ice unless otherwise specified and 50 mM sodium phosphate buffer, ph 7.0, was used throughout.

The crude sample was applied to a column (1.5 x 20 cm) of CM-Sephalose Cl-6B (Pharmacica Fine Chemicals, Uppsala) conditioned with the buffer. The column was washed exhaustively with the buffer until absorbance at 280 nm became less than 0.01 and then was eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer at a flow rate of 20 ml/hr. Each fractions of 5.9 ml was collected. Proteins were monitored by absorbance at 280 nm and by SDS- polyacrylamide gel electrophoresis (SDS-PAGE) as described below. Fractions containing proteins were pooled and concentrated approximately 10 times by centrifugation under the reduced pressure, and then dialyzed against 50 mM phosphate buffer, pH 7.0, overnight.

Sephacryl-400 Gel permeation chromatography

The crude sample and purified glue protein-1 of peak 3 from CM-Sephalose CL-6B column were applied on a Sephacryl-400 Gel permeation column (1.5 x 92 cm). The column was equilibrated and eluted with 50 mM Phosphate buffer at the rate of 16 ml/hr and the absorbances were monitored at 280 nm. Thyroglobulin, (M_r 669,000), *Bombyx mori* vitellin, (M_r 420,000) and catalase, (M_r 232,000) were used to calibrate the column.

High Performance Liquid Chromatography

The final step of purification was performed by high performance liquid chromatography (HPLC). Samples (50 ul containing about 100 ug proteins) were injected into a molecular sieving column (0.7 x 25 cm, DC-250 Shimazdu-DuPon) equilibrated with the buffer containing 0.1 M Na_2SO_4. The proteins were eluted with the same buffer at a flow rate of 1 ml/min at room temperature. The fractions containing glue protein were pooled and concentrated as above.

Polyacrylamide gel electrophpresis

The dialyzed samples from CM-Sephalose chromatography were mixed with the same volume of SDS-sample buffer and heated in a boiling water bath for 5 min. After a brief centrifugation, the supernatant fraction (40 ul containing about 50 ug proteins) was applied to a 7.5% 1-mm slab gel and electrophoresed according to the method of Laemmli (1970). Gels were stained for proteins with Coomassie brilliant blue R-250 and then destained to realize protein bands. In some experiments, silver staining method was also applied to detect the proteins bands according to the instruction of the supplier.

Molecular weight estimation

The molecular weight of denatured proteins was estimated by SDS-PAGE by comparing the relative mobilities of the standards proteins which were co-electrophoresed. The marker proteins, "DAIICHI" II, were obtained from Daiichi Pure Chemicals Co., Tokyo, and Molecular Weight Marker Kit, Catalog No. MW-SDS- 17S from Sigma Chemical Co., U.S.A.

Molecular weight of native form was determined by sucrose gradient centrifugation and HPLC and Sephacryl-400 Gel permeation chromatography as decribed above.

Amino acid analysis

The purified protein was hydrolyzed with 6 M HCl at 110°C for 24 hr in evacuated and sealed tube. The hydrolyzates were analyzed with an automatic amino acid analyzer (JEOL JIC-8AH).

Sequencing of NH_2-terminal amino acid

The purified proteins were exhaustively dialyzed against distilled water. Amino acid sequence anaylsis was performed by automated Edman degradation using an Applied Biosystems 477A Protein Sequencer equipped with a 120A PTH-analyzer.

Preparation of antibody

The purified glue protein-1 (5 mg) was emulsified with Freund's complete adjuvant (Difco Laboratories, Detroit) and was injected subcutaneously into the back of a rabbit. After 5 weeks later booster injection (1 mg) was administered. Blood was collected 1 week later and serum was stored at -20°C.

Western blotting

Immunoblotting was carried out according to the method of Burnett (1981) with some modifications. The proteins separated by SDS-PAGE, were electrophoretically transferred to nitrocellulose membrane filter. The filter was first treated with anti-glue protein-1 serum diluted to 1/10,000, and then with peroxidase- conjugated anti-rabbit IgG goat IgG. The immunoactive proteins were visualized by incubating the filter with Konica immunostain (HRPIS-50B) according to the instruction of the supplier (Konica Co., Tokyo).

RESULTS

Purification of *Bombyx mori* glue protein

Crude sample was used for purification. Purification was carried out by CM-Sephalose CL-6B chromatography, monitored the protein contents at 280 nm and separated polypeptide by 7.5% and 15% SDS-PAGE (Figure 1). The fractions from CM-Sephalose column always gave three major peaks, peak 1, 2 and 3, as shown on Figure 1A. The proteins from peak 1, 2 and 3 were eluted at 0.09-0.1 M, 0.21-0.22 M, and 0.26-0.29 M, respectively. The fraction of peak 1 showed two major bands, glue protein-1 (GP-1), glue protein- 2 (GP-2), and serverals minor bands on 7.5% and 15% gel (lane 1 Figure 1B; lane 1-5 Figure 1C).

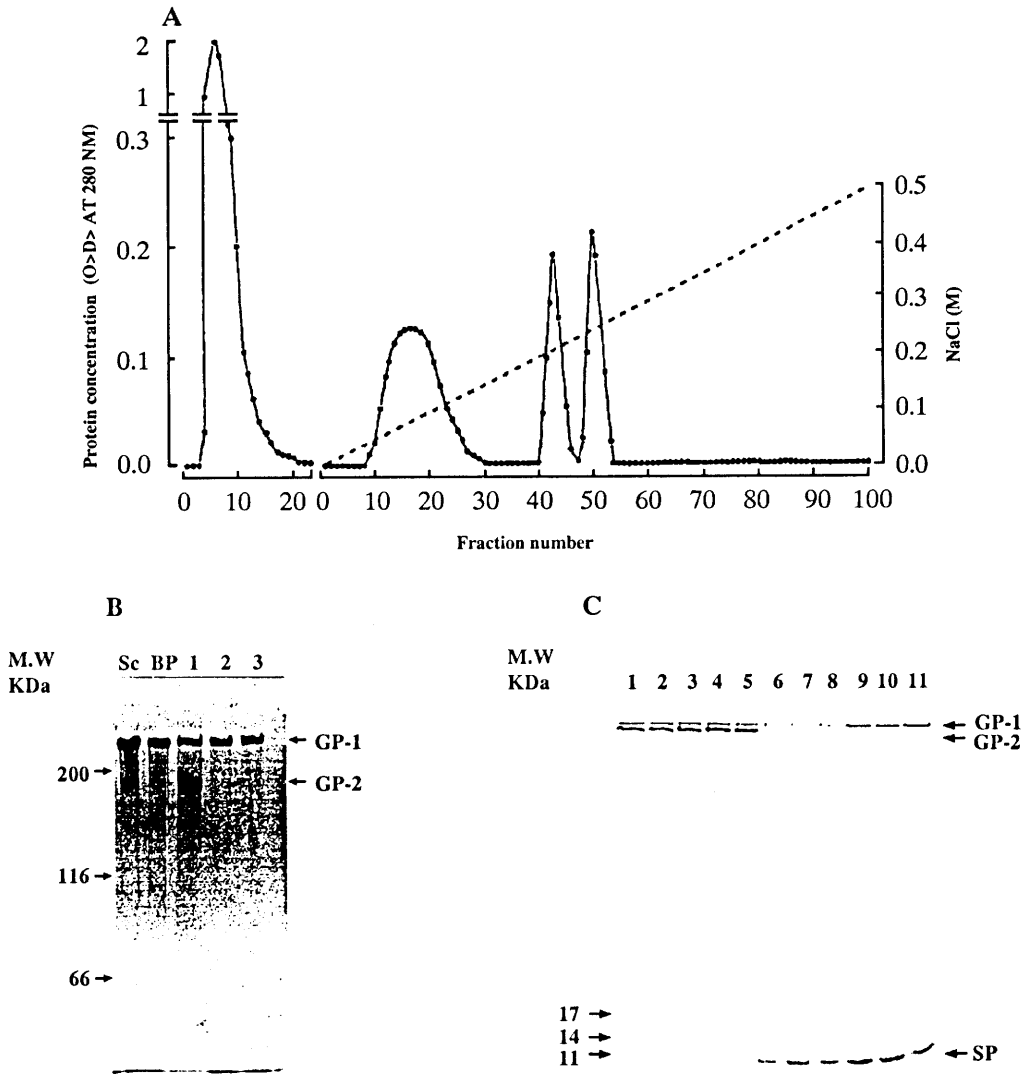

Figure 1 CM-Sephalose CL-6B chromatography of secretion of colleterial glands (panel A). Proteins were eluted with a linear gradient of 0-0.5 M NaCl in 50 mM phosphate buffer, pH 7.0 and protein content was monitored at 280 nm. Aliquots of peak 1,2 and 3 were concentrated, dialyzed and separated by SDS-PAGE using 7.5% gel (panel B) and 15% gel (panel C). GP-1, glue protein-1; GP-2, glue protein-2; Sc, crude secretion; Bp, Buffer eluted; Sp, small peptide.

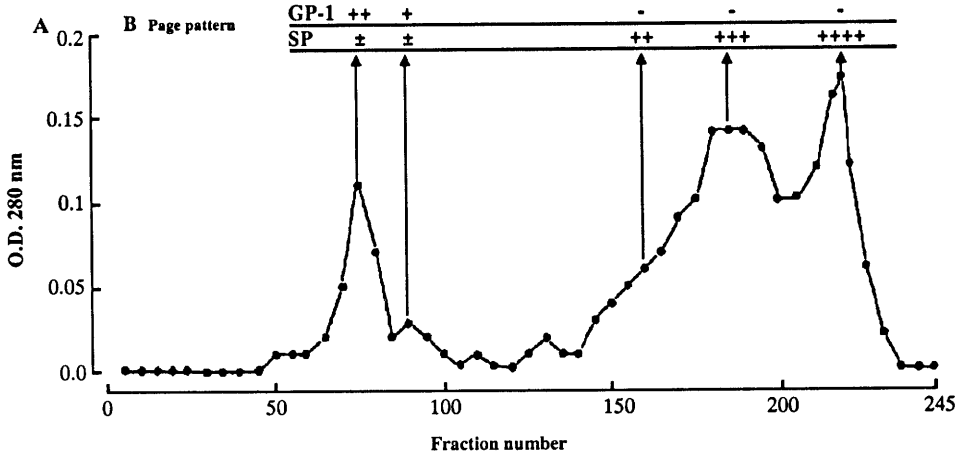

Figure 2 Sephacryl-400 Gel permeation chromatography of peak 3 from CM-Sephalose CL-6B chromatography (panel A.). The column was eluted with 50 mM phosphate buffer, pH 7.0. Protein content was followed by measuring the absorbance at 280 nm. Several fractions were subjected to 15% SDS-PAGE and relative intensity of GP-1 and SP were shown in the upper panel B.

Table 1 Amino acid composition of the *Bombyx mori* purified proteins from CM-Sephalose CL-6B chromatography (mole %).

Amino acid	Peak 1 GP-2	Peak 2 GP-1	Peak 3 GP-1
Asp	12.0	8.3	8.8
Thr	5.2	3.5	3.6
Ser	4.9	4.2	4.6
Gln	16.1	32.8	31.0
Pro	15.0	8.7	4.6
Gly	9.8	25.0	26.8
Ala	4.9	2.6	2.2
Cys	5.5	0.5	0.5
Val	5.0	1.6	2.2
Met	0.1	0.2	0.2
Ile	2.3	1.2	1.8
Leu	3.8	2.0	2.5
Tyr	4.0	1.1	1.8
Phe	0.5	0.5	0.6
His	0.6	2.0	2.6
Lys	5.7	4.1	4.8
Arg	4.8	1.6	1.5

Peak 2 and peak 3 gave a single band, GP-1, on 7.5% gel (lane 2,3 Figrue 1B) but on 15% gel small peptide, SP, was detected (lane 6-8 of peak 2 and lane 9-11 of peak 3 in Figure 1C). This peptide could not be detected in peak 1. The polypeptide of peak 2 and 3 had identical electrophoretic mobility and relatively high contents of GP-1. GP-2 was detected at high intensity in peak 1.

In order to separated SP from GP-1, aliquots from speak 3 were pooled, concentrated and performed by Sephacryl-400 Gel permeation chromatography, and later the banding pattern was determined by 15% gel (Figure 2).

Molecular weight determination

The apparent molecular weight of the GP-1 and GP-2, on the basis of their mobility on SDS-polyacrylamide gel electrophoresis were 240 and 190 KDa respectively (Figure 1B). In some case, a small peptide was determined on 15% SDS-PAGE with an apparent molecular weight of about 10 KDa (Figure 1C).

By using Sephacryl-400 column, an apparent molecular mass of native protein was estimated to be 7,000 KDa for GP-1, 1,700 KDa for GP-2 and 26 KDa for SP (Figure 3)

Amino acid composition of *Bombyx mori* glue protein

The amino acid composition of the purified glue protein was given in Table 1. It is novel in its high content of glutamic acid. *Bombyx mori* glue protein also showed remarkably high amounts in glycine and very low in methionine. The composition of glue protein was predominanted by acidic amino acid indicating its strong negative charges.

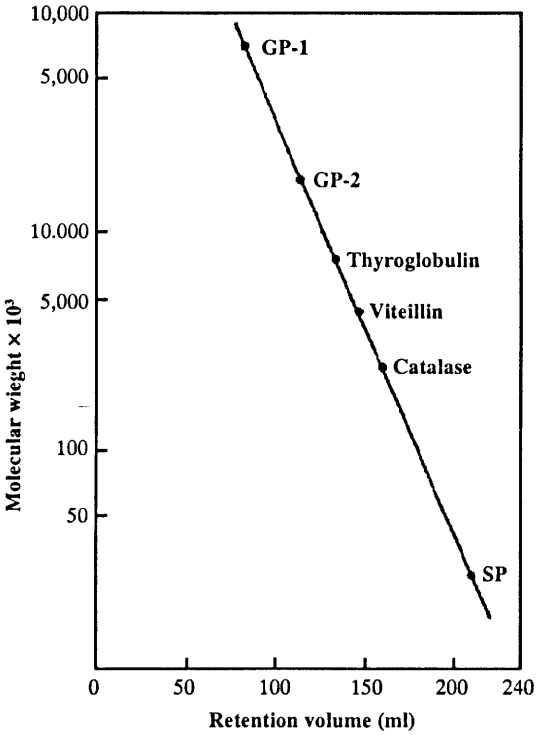

Figure 3 Estimation of Molecular Weight of glue proteins by gel permeation on Sephacryl-400. Elution was performed with 50 mM phosphate buffer, pH 7.0.

	1	5	10	15	20
Peak 2	M-L-G-X-L-K-K-D-X-N-V-P-I-Y-D-K-X-A-N-K				
Peak 3	T-L-G-W-L-S-K-D-Q-N-N-P-I-Y-Y-K-N-A-N-V				
	Y P Y N			Q Q	

Figure 4 NH_2-terminal amino acid sequences of *Bombyx mori* glue protein-1. X indicates residues whose identity could not be determined.

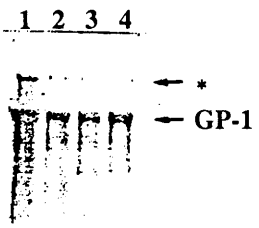

Figure 5 Immunoblot of the purified fractions from CM-Sephalose column by glue protein-1 serum and IgG. lane 1, crude sample; lane 2, aliquots of peak 1; lane 3, aliquots of peak 2; lane 4, aliquots of peak 3; *, aggregated glue protein-1.

NH_2-terminal amino acid sequence

The NH_2-terminal amino acid sequences of the purified glue protein-1 from peak 2 and peak 3 were shown in Figure 4. They showed quite similar in sequences of about 60-75%. Approximately 55% is composed of polar amino acid in both sequences.

Immunological properties

In an immunoblotting experiment, purified GP-1 antiserum which was raised against the purified protein fractions from CM-Sephalose column occurred mainly to the GP-1 (Figure 5).

DISCUSSION

Identification and characterization of the secretory proteins from female accessory glands have been accomplished in several varieties of insect species. (Pau *et al.*, 1971; Kramer *et al.*, 1973; Lenoble and Denlinger 1982; Dallai *et al.*, 1986). In *Bombyx mori*, although the structural, cytological and developmental studies have been done (Suzuki and Tsujita, 1937; Omura, 1938; Arisawa and Fugo, 1990; Amornsak *et al.*, 1992), there is only a limited information on chemical components of secretion of silkworm colleterial glands (Arisawa and Fugo., 1990; Amornsak *et al.*, 1992). Almost no experiment is carried out on the biochemical properties of the silkworm glue proteins other than those of the other insects. A major problem during purification is that glue protein is sticky, aggregated and may lose some properties during storage.

The glue protein purified from *Bombyx mori* colleterial gland by CM-Sephalose CL-6B column gave two major proteins, GP-1 and GP-2, on 7.5% polyacrylamide gel electrophoresis and SP was detected on 15% SDS-PAGE in peak 2 and 3 except for in peak 1. It is possible that SP was converted from GP-1. Purification of glue proteins lead to a product of high purity of GP-1 but it was recovered quite low of only 19-20% and 22-25% from peak 2 and peak 3 respectively while 53% recovered from peak 1. This may due to some GP-1 being first washed out by the buffer.

GP-1 from peak 2 and 3 were estimated to be the same molecular weight but with different charges (Figure 1). Using SDS-PAGE, an estimated molecular weight of the predominance glue protein was found to be 240 KDa of very large molecular weight compared to the other proteins of the same organs of other insects which ranged between 13-47 KDa. Secretory proteins in cockroach, mantid, medfly and tsetse fly have molecular weight about 13-39, 43, 13-38 and 47 KDa, respectively (Pau *et al.*, 1971; Kramer, *et al.*, 1973; Dallai *et al.*, 1986; Osir *et al.*, 1991). However in cecropia silkmoth, the molecular weight of the major protein is found to be about 160 KDa which is quite the same size as that of the silkworm

Bombyx mori (Grayson and Berry, 1974).

The amino acid composition of the silkworm glue protein was found to be different from the other insect species which showed remarkably high contents of glutamic acid and glycine (Table 1) while high amount of aromatic amino acid in the cockroach and the tsetse fly were observed (Pau *et al.*, 1971; Osir *et al.*, 1991). It is possible that the function of protein from colleterial glands of these insects are different. In the cockroach, they lead to a formation of sclerotin of the egg-case, ootheca, and for puparium formation in the tsetse fly. Contrasting to silkworm as well as cecropia silkmoth, the secretory proteins would glue eggs to a substrate or may form a protective coat over the eggs during oviposition. The glue protein of both domestic silkworm and cecropia silkmoth showed a predominance of acidic amino acid but lack of aromatic amino acid while the secretory protein from the left colleterial gland in the cockroach showed more basic amino acid. The amino acid composition of the silkworm glue protein is also similar to that of the mantid (Kramer *et al.*, 1973) in having high amount of glutamic acid and glycine. However, their functions are quite different since in the mantid secretory proteins were used for tanning and hardening of the egg-case instead of sticking the eggs to the substrate as that of the silkworm.

It is interesting to learn that the NH_2-terminal amino acid sequence consisted only 5% of glutamic acid and glycine, which is not in corresponded to amino acid composition of the whole molecule. There is a possible explanation that the region containing glutamic acid and/or glycine may locate at the other position of the glue protein molecule.

The work presented here can facilitate further biochemical studies concerning the physiological mechanism system in insect and helps in developing synthetic chemicals of similar nature.

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