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CELL-FREE PROTEIN SYNTHESIS BY POSTERIOR SILK GLAND POLYRIBOSOME

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Polyribosome was prepared from the posterior gland of silkworms, where silkfibroin is specifically synthesized. Coupled with the corresponding enzymes, amino acids, ATP and GTP, silkfibroin was produced in a cell-free system. The production of silkfibroin was monitored by SDS polyacrylamide gel electrophoresis of the dansylated polypeptides. The polysome fraction was adsorbed on DEAE-Sephadex. Using the immobilized polysomes, a genetic information-transducing bioreactor for producing a specified protein was assembled. The promising features of this bioreactor are discussed.

INTRODUCTION

Considerable attention has been paid to the development of a selective *in vitro* process for protein synthesis that is initiated by genetic information. Our concerns have been directed to the reconstitution of protein biosynthetic systems for the purpose of continuous protein production. Cell-free protein syntheses studied in various ways have provided information that ribosomes, mRNA, and several soluble factors should be properly assembled in such a system.

The posterior gland of the silkworm *Bombyx mori*, which consists of a single cell type, undergoes extensive mRNA synthesis during the first three to four days of the last larval instar (1). The mRNA synthesis is followed by the selective production of silkfibroin. The posterior silk gland offers an ideal model of a bioreactor for producing a specific protein. In a previous investigation, the posterior silk gland was immobilized in a gel matrix with retention of its function (2). The immobilized posterior silk gland was applied to the production of silkfibroin.

The present investigation was undertaken to show the possible incorporation of posterior silk gland polyribosome in a bioreactor for protein synthesis based on mRNA. Silk protein was produced in a cell-free system that contained the posterior silk gland polyribosomes, mRNA, and soluble factors. Special care was paid to the use of the polyribosomes as an

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information transducing organelle. In a further investigation, the immobilization of polyribosome on a solid support was attempted.

MATERIALS AND METHODS

Materials

Acrylamide, *N,N'*-methylene bisacrylamide (BIS), dansyl chloride, sodium dodecyl sulfate (SDS), ammonium persulfate, and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) were purchased from Tokyo Kasei Co., Ltd. (Tokyo), GTP and polyuridylic acid [Poly(U)] from Yamasa Shoyu Co., Ltd. (Tokyo), ATP from Kyowa Hakko Co., Ltd. (Tokyo), and sucrose (RNase free) from Nakarai Chemicals Co., Ltd. (Kyoto). Silkworm larvae were generously supplied by Dr. N. Yoshitake and Dr. M. Kobayashi (Faculty of Agriculture, University of Tokyo). The larvae were the hybrid of *Bombyx mori* strains *Shunrei* and *Shogetsu*, reared on fresh mulberry leaves at room temperature in the laboratory, and sacrificed on the 5th or 6th day of the 5th instar.

Preparation of Posterior Silk Gland Polyribosome

The posterior silk gland polysome was prepared according to Shimura's method (3). The larvae were dissected on the fifth to the sixth day of the fifth instar. The posterior parts of the paired glands were collected and rinsed in 1.15% KCl to remove adhesive fat and tracheas. The preparation was frozen for storage.

The frozen gland was cut into 5-mm pieces and homogenized in 2 vol of buffer of pH 8.0 containing 5 mM $MgCl_2$, 50 mM KCl, 400 mM sucrose, and 50 mM tris-HCl (Buffer I) at 3000 rpm with a Waring blender for 2 min. The homogenization was finished before the frozen gland thawed. The homogenate was filtered on two layers of cheese cloth. The filtrate was centrifuged at $700 \times g$ for 10 min. The supernatant was centrifuged at $20,000 \times g$ for 30 min to precipitate the polysome fraction. The supernatant was again centrifuged at $105,000 \times g$ for 90 min, and was used as the soluble enzyme fraction.

The polysome pellet fraction was dispersed in 2 vol of buffer I and homogenized in a Tefron-glass homogenizer with a few strokes, which was followed by the addition of 20% deoxycholic acid to bring the final concentration to 1%. The resulting solution was again homogenized in a Teflon-glass homogenizer with a few strokes. The homogenate was then centrifuged at $14,000 \times g$ for 30 min, and 2 vol of buffer I was added to the precipitated pellet. The dispersion was again homogenized in a Teflon-glass homo-

genizer and then sonicated at 20 KHz for 3 min. Finally, the homogenate was centrifuged at $14,000 \times g$ for 20 min. The precipitated pellet was made up of large size polyribosomes.

Contamination by soluble proteins in the polysome fraction was checked by following an absorbance ratio of 260 and 280 nm [A_{260}/A_{280}]. The polysome fraction gave a volume for A_{260}/A_{280} of 1.7. It is accepted in general that A_{260}/A_{280} of purified polysomes ranges from 1.6 to 1.8. The polysome fraction prepared by the present method is considered to be sufficiently purified.

The polysome fraction is believed to contain the conjugates of silk fibroin mRNA [mol. wt. 5,700,000 (4)] and 80S ribosomes. In order to ascertain the presence of mRNA, the poly(A) moiety of silk fibroin mRNA was determined by affinity chromatography on poly(U)-Sepharose 4B, because the silk fibroin mRNA is specified by the involvement of the poly(A) moiety at the 3'-terminal. The procedure for preparation of poly(U)-Sepharose 4B is described in the following section.

One ml of the polysome fraction was applied to poly(U)-Sepharose 4B for affinity chromatography. Elution was carried out with an isotonic buffer of pH 7.5, a hypertonic buffer of pH 7.5, and a 1 M acetate buffer of pH 3.2 successively. In a similar manner to the above, chromatography on Sepharose 4B was performed as a control. Figure 1 shows the elution patterns of the polysome fraction on Sepharose 4B and poly(U)-Sepharose 4B. A marked difference in the elution pattern arose in the range of fraction numbers 3–10. The ingredients of fraction numbers 3–5 on Sepharose 4B were eluted in the range of 7–10 on poly(U)-Sepharose 4B, which should result from the interaction of poly(A) with poly(U). Affinity chromatography indicated that the polysome fraction might contain the poly(A)-containing mRNA. From the chromatogram, the amount of the poly(A)-containing composite was estimated as 2.4 A_{260} units.

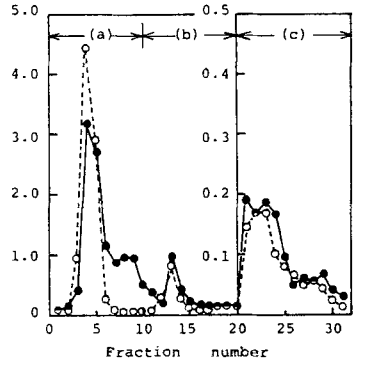

FIG. 1. Column chromatography of the posterior silk gland polyribosomes on poly(U)-agarose. The polyribosome fraction (50 mM KCl, 5.0 mM $MgCl_2$, and 50 mM tris-HCl buffer at pH 8.0) was layered on the poly(U)-agarose column and then eluted with (a) Buffer I as an isotonic buffer; (b) hypertonic buffer, 2 M NaCl, 6 M urea, and 50 mM tris-HCl (pH 7.5); and (c) 1 M acetate buffer (pH 3.2). Each eluate was monitored photometrically at 260 nm. The elution pattern for poly(U)-agarose is shown in solid circles, whereas that for agarose in shown in open circles.

Preparation of Poly(U)-Sepharose 4B

Sepharose 4B (70 ml) was thoroughly washed with deionized water and the final volume was brought to 30 ml. A cyanogen bromide (CNBr) solution (6 g/100 ml) was added dropwise to the Sepharose 4B suspension, while the pH was maintained at 11 with 8 N KOH. The suspension was allowed to stand at pH 11 for 10 min, and was then filtered in a glass funnel. The filtrate was washed with 500 ml of ice-cooled distilled water.

Ten ml of the CNBr-activated Sepharose 4B was washed several times with 0.1 M potassium phosphate buffer, pH 7.5, and finally suspended in 10 ml of the buffer. To this Sepharose 4B suspension was added 1 ml of poly(U) solution (4.7 mg/ml phosphate buffer) at 4°C. The coupling reaction was completed within 1 h by monitoring photometrically the unreacted poly(U). The resultant Sepharose 4B was filtered and washed with the elution buffers. The elution buffers used were (*a*) 10 mM phosphate buffer of pH 7.5, containing 10 mM EDTA, 90% formamide, and 0.2% lauryl sarcosine; (*b*) 10 mM tris-HCl of pH 7.5, containing 0.15 mM NaCl and 1.5 mM $MgCl_2$; (*c*) 10 mM tris-HCl of pH 7.5, containing 0.7 M NaCl, 10 mM EDTA, and 25% formamide, and (*d*) 1 M acetate buffer of pH 3.2.

Cell-Free Protein Synthesis

Cell-free protein synthesis was performed with the polysome fraction and the soluble enzyme fraction in the presence of amino acids, ATP and GTP as shown in Tables 1a and 1b. The amino acid solution was made according to the amino acid content of silkfibroin as given in Table 1b. The

TABLE 1a. **Culture Medium for Cell-Free Fibroin Synthesis by Silk Gland Polysomes**

Ingredient	Control	Synthesis
Polysome (A_{260})[a]	110	110
Supernatant (ml)	4.0	4.0
ATP (mM)	4.0	4.0
GTP (mM)	2.0	2.0
Amino acid mixture (ml)[b]	0	2.0
KCl (mM)	50	50
$MgCl_2$ (mM)	5.0	5.0
Sucrose (mM)	400	400
Dithiothreitol (mM)	2.0	2.0

[a] In the polysome fraction are contained 30 U of 160S polyribosomes and 80 U of 420S polyribosomes.
[b] The content of the amino acid mixture is shown in Table 1b.

TABLE 1b. **Content of the Amino Acid Mixture (mg/100 ml) Used for Protein Synthesis**

Glycine	89	Phenylalanine	2.0
Alanine	43	Glutamic acid	3.0
Serine	23	Isoleucine	3.0
Tyrosine	11	Proline	1.0
Valine	5.0	Cysteine[b]	1.0
Threonine	2.0	Histidine	1.0
Lysine[a]	5.0	Tryptophane	1.0
Leucine[a]	2.5	Methionine	1.0
Arginine	2.5	Asparagine	1.0
Aspartic acid	4.0	Glutamine	1.0

[a] HCl salt.
[b] Added before use.

pH of the ATP and the GTP solutions was adjusted to pH 8 with 1 N KOH before use. All the equipment was sterilized at 150°C for 3 h. The amino acid solution and Buffer I (containing 50 mM tris-HCl at pH 8.0, 5 mM $MgCl_2$, 50 mM KCl and 400 mM sucrose) were heated at 100°C for 100 min to avoid contamination by RNase and bacteria. A medium without any amino acids was used as a control. The reaction was continued at 30°C for 4 h.

SDS-Polyacrylamide Gel Electrophoresis of Dansylated polypeptides

SDS-polyacrylamide gel electrophoresis was performed with dansylated polypeptides according to Kato et al. (5). After 2 and 4 h of cell-free protein synthesis, the polypeptides contained in the reaction mixture were dansylated as follows; 0.2 ml of the opalescent reaction mixture was made transparent by the addition of 0.2 M phosphate buffer, pH 8.3, in 0.2% SDS. This was reacted with 40 μl of 0.3% dansyl chloride acetone solution at 50°C for 30 min. The resulting solution was reduced by 40 μl of mercaptoethanol at 50°C for 30 min. Acrylamide gels were prepared in tubes (5 × 100 mm) as described by Weber et al. (6). The labeled polypeptides were then layered gently on the top of the gels (4% acrylamide, 0.11% BIS) in the volume range 50–100 μl. Carbonate buffer (0.1 M), pH 8.8, containing 0.5% SDS was used as the tray buffer. Electrophoresis was carried out at a current of 5–8 mA per one gel tube for 4–8 h. After electrophoresis, each gel was soaked in 0.5 M phosphate buffer, pH 7.5, to remove dansylated low molecular compounds in order to obtain a clear electrophoretic pattern. The gels were exposed to light at 360 nm for visualization of the electrophoretic pattern.

Immobilization of Silk Gland Polyribosome

The posterior silk gland polyribosome was immobilized on the matrix by adsorption. DEAE-Sephadex was packed in a column (20 × 80 mm) and equilibrated with Buffer I. Two ml of the polysome fraction was applied to the column. After sufficient adsorption, the leakage of the adsorbed polysome was tested with several elution buffers. The elution buffers used were (*a*) isotonic buffer, 0.15 M NaCl, 1.5 mM $MgCl_2$, and 10 mM tris-HCl (pH 7.5); (*b*) isotonic buffer containing EDTA, 0.15 M NaCl, 10 mM EDTA (2Na), and 10 mM tris-HC1 (pH 7.5); (*c*) hypertonic buffer, 2 M NaCl, 6 M urea, 10 mM EDTA (2Na), and 50 mM tris-HCl (pH 7.5); and (*d*) 1 M acetate buffer (pH 3.2).

Cell-free Protein Synthesis Using Immobilized Polysome

The DEAE-Sephadex-bound polysome (5.4 ml, 118 A_{260} U of polysome) was packed in a column (20 × 80 mm). Three ml of the soluble enzyme fraction, 50 ml of 10 mM tris-HCl at pH 8.0, containing 2.0 mM dithiothreitol, 1.6 mM ATP, 1.6 mM GTP, 5.0 mM $MgCl_2$, 50 mM KCl, and 2.0 ml of the amino acid mixture described in Table 2 were added to the column. After 7 h incubation at 30°C, the reaction mixture was eluted stepwise with (*a*) isotonic buffer, (*b*) 5 M NaCl (twice), and (*c*) 1 M acetate buffer of pH 3.2. The eluate was collected in 0.1-ml fractions, and labeled with dansyl chloride for SDS gel electrophoresis.

RESULTS

Silkprotein Synthesis in the Cell-Free System

Cell-free silk gland polysomes, the soluble factors, amino acids, and energy sources were described above. The products were determined by SDS gel electrophoresis after 2 and 4 h of reaction. The reaction mixture gave five bands (*a–e*) as shown in Fig. 2. The fluorescent intensity of band *a* increased sharply with reaction time, while those of the other bands decreased. Furthermore, the molecular weight of the band *a* substance was estimated as 3000,000–400,000 from the electrophoresis of human plasma fibrinogen (mol. wt. = 400,00).

These results indicate that band *a* of the electrophoretic pattern should be attributed to that of silkfibroin. The control system, which contained no amino acids, gave a slightly fluorescent band *a*, as presented in Fig. 2. The band, however, remained constant in fluorescent intensity throughout the reaction. It is considered that band *a* of the control system might be caused

TABLE 2. **Culture Medium for Fibroin Synthesis by Immobilized Silk Gland Polyribosomes**

Ingredient	Control	Synthesis
Polysome (A_{240})	118	118
Supernatant (ml)	3.0	3.0
ATP (mM)	0	1.6
GTP (mM)	0	1.6
Amino acid mixture (ml)[a]	0	2.0
KCl (mM)	50	50
$MgCl_2$ (mM)	5.0	5.0
Sucrose (mM)	400	400
Dithiothreitol (mM)	2.0	2.0

[a]The content of the amino acid mixture is shown in Table 1b.

by the contaminated silkfibroin in the soluble factors. A marked increase in the fluorescent intensity of band *a* as compared with the control system reveals that silkfibroin was synthesized in the cell-free system.

Bands *b*, *c*, *d*, and *e* disappeared with reaction time both in the control and synthetic systems. This strongly suggests that these bands can be attributed to RNAs. From electrophoretic patterns, bands *b*, *c*, *d*, and *e* are ascribable to 28 S, 18 S, 5 S ribosomal RNAs, and transfer RNA, respectively. The dissipation of these bands may result from the degradation of RNAs by the action of RNase.

Silkfibroin Synthesis by the Immobilized Polysome

The polysome fraction was adsorbed to DEAE-Sephadex. To ascertain the feasibility of the gel as a supporting matrix for polyribosome immobilization, the polysome fraction was eluted with (*a*) isotonic buffer at pH 7.5, (*b*) isotonic buffer at pH 7.5 containig EDTA, (*c*) hypertonic buffer at pH 7.5, and (*d*) acetate buffer at pH 3.2. Figure 3 shows the elution pattern.

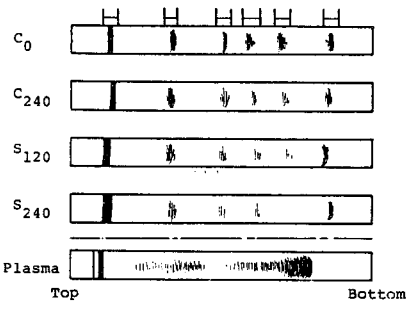

FIG. 2. SDS gel electrophoresis for cell-free protein synthesis. Composition of the cell-free reaction mixture is described in Tables 1a and 1b. After incubation for 120 and 240 min, an aliquot of the sample mixture was dansylated, reduced, and electrophoresed. No amino acids were contained in the control mixture. The letters C and S stand for the control and synthesis systems, respectively, and the suffix numbers correspond to incubation time. In this electrophoresis, plasma proteins were used as an indication of electrophoretic patterns.

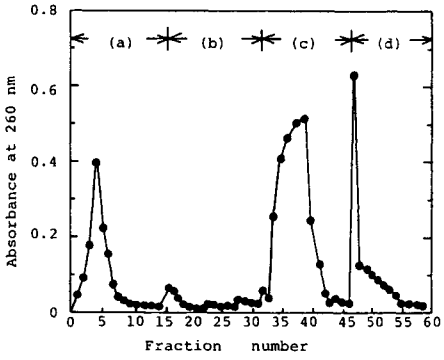

FIG. 3. Adsorption and desorption of the polyribosome fraction on DEAE-Sephadex. Two ml of the polyribosome fraction was layered on DEAE-Sephadex, and then passed over with (a) isotonic buffer, 0.15 M NaCl, 1.5 mM $MgCl_2$, and 10 mM tris-HCl (pH 7.5); (b) isotonic buffer containing EDTA, 0.15 M NaCl, 10 mM EDTA (2 Na), and 10 mM tris-HCl (pH 7.5); (c) hypertonic buffer, 2 M NaCl, 6 M urea, 10 mM EDTA (2Na), and 50 mM tris-HCl (pH 7.5); and (d) 1 M acetate buffer (pH 3.2).

When the fraction was eluted with (*a*), a considerable absorbance at 260 nm was observed. However, the absorbance ratio A_{260}/A_{280} of this peak was around 1.2. This indicates that the major components of the peak were proteins, and no appreciable leakage of the polysome fraction resulted from the isotonic buffer (*a*), because the absorbance ratio for the polysome fraction was about 1.7. In contrast, the hypertonic buffer and acetate buffer caused the adsorbed polysomes to be eluted. This was ascertained by monitoring the absorbance ratio. Therefore, it is suggested that immobilized polysomes can be prepared and may be used under isotonic conditions.

The polysomes (118 U) immobilized on DEAE-Sephadex were placed in contact with the soluble factors, the amino acid mixture, and ATP and GTP as presented in Table 2. The minor components, which were not retained in the gel matrix with the isotonic buffer (*a*), were regarded a part of the supernatant fraction, and were added to the reaction system. The content of the amino acid mixture is shown in Table 1b. The reaction system was stirred every 30 min, and was allowed to stand 420 min. The reaction mixture was eluted sequentially with (*a*) buffer I, (*b*) 5 M NaCl (twice), and (*c*) 1 M acetate buffer (pH 3.2). Each 0.1-ml fraction was dansylated and reduced with mercaptoethanol for SDS polyacrylamide gel electrophoresis. The electrophoretic patterns presented in Fig. 4 as those of the control system,

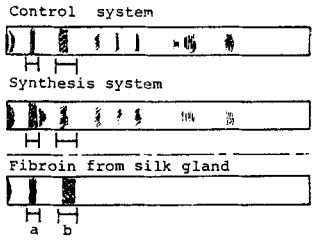

FIG. 4. SDS gel electrophoresis of the protein synthesis system coupled with the immobilized polyribosomes. Composition of the reaction mixture for fibroin synthesis using immobilized polyribosomes is shown in Table 2. After 420 min of incubation, the reaction mixture was eluted with (a) buffer I, (b) 5 M NaCl (twice), and (c) 1 M acetate buffer (pH 3.2). Each eluate was fractionated at 0.1 ml, dansylated, and then electrophoresed. For the control system, the incubation time was zero, whereas for the synthesis system, the incubation time was 420 min. In this case, fibroin extracted from the middle silk gland was used as an authentic sample.

synthetic system, and the silkfibroin extracted from the most posterior part of the middle silk gland, where silkfibroin is stored. Each electrophoretic pattern of the control and synthetic systems was superimposed to make comparison between the two systems easier. An authentic sample of silkfibroin from the middle gland gave a very weak subband *b* besides the major band *a*. The subband resulted from the involvement of a small molecular weight minor component of fibroin. From comparative studies of band *a* between the control system, where neither amino acids nor energy sources were contained, and the synthetic system, it was concluded that silkfibroin was synthesized by incorporating amino acids with the decoding action of the immobilized polysomes, because wider and more dense fluorescence was observed in band *a* of the synthetic system than in band *b* of the control system.

DISCUSSION

Amino acids have been incorporated in various systems for cell-free protein synthesis. Protein synthesis has been commonly monitored by counting radioactive markers in the systems because of the excellent sensitivity and simplicity of RI methods. Therefore cell-free protein synthesis has been performed in the order of picogram concentrations. The present investigation was aimed primarily at the protein synthesis in the concentration range of at least nanograms. SDS gel electrophoresis with fluorescence labeling should be best suited to this purpose. Silkfibroin was successfully synthesized by assembling silkgland polysomes, soluble enzyme factors, energy sources, and amino acids. The synthesized fibroin was believed to possess a molecular weight of 300,000–400,000, and to amount to more than 1 ng in the 4-h reaction. Silkfibroin has been partially synthesized in several cell-free systems (3,4,7). The molecular weights of these products have been far less than 300,000. In this investigation silkfibroin is considered to be totally synthesized.

Cell-free protein synthesis may be restricted seriously by the short lifetime of mRNA and the contamination of RNase. It has been well described that eukaryotic mRNA is much more stable than prokaryotic mRNA, because the 5'-terminal confronting structure (8) and the 3'-terminal poly(A) chain prevent eukaryotic mRNA from degrading by RNase. The mRNA from the posterior silk gland seemed to be fairly stable, which led to fibroin production in nanogram concentrations. The immobilized ribosomes may be reusable, providing considerable advantage for the construction of a bioreactor which continuously produces a specific protein on the basis of the corresponding mRNA information. We believe that the present approach is promising in relation to the development of this frontier in bioengineering.

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