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PREPARATION OF RECOMBINANT BACULOVIRUS DNA CODING FOR β-GALACTOSIDASE OF *Escherichia coli* AND THE EXPRESSION PRODUCT IN *Bombyx mori* LARVAE

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A method has been developed for preparing recombinant baculovirus that encoded foreign protein (using β-galactosidase of Escherichia coli *as an example) directly in* Bombyx mori *larvae (silkworm) and avoids cultivation and the use of a cell line, which are very difficult and require expensive equipment and reagents. This significantly reduces the cost of the recombinant protein without loss of biological properties.*

Key words: recombinant plasmid DNA, β-galactosidase of *Escherichia coli*, *Bombyx mori*.

One of the main goals of biotechnology is to prepare recombinant proteins that have the same structure and functionality as the natural analogs. Several expression systems are known that fulfill this expectation more or less successfully. Satisfactory results were obtained in expression systems based on bacteria and yeast. However, not all proteins of eukaryotes that are produced in these systems possess biological activity comparable to that of their cellular analogs. On the other hand, an expression system based on mammalian cells can produce recombinant proteins that correspond antigenetically and functionally to the natural analogs. The single and important drawback of this system is the high cost of the recombinant proteins. The baculovirus—insect-cell expression system combines the necessary biological activity and cost-efficiency of recombinant proteins, which favorably distinguishes it from the systems mentioned above.

Proteins that are encoded by various genes are obtained in high yield in vector systems based on baculovirus in various cell lines [1]. These vector systems were useful for basic research and for industrial exploitation. Numerous examples of the successful and effective expression of biologically active vertebrate proteins are known.

Nuclear polyhedrosis viruses (NPV) *Autographa californica*, *Spodoptera frugiperda*, *Bombyx mori*, and *Trichoplusia ni* are used most often as the transfecting vectors. These are rather species-specific and contain a strong promoter of the polyhedrin gene, under the control of which they assimilate foreign cDNA.

The following strategy is usually used to prepare recombinant proteins in the baculovirus expression system: 1) production of recombinant plasmid DNA in which the cDNA of the gene coding for the necessary recombinant protein is under the control of the polyhedrin gene promoter; 2) homologic recombination between wild NPV and recombinant plasmid DNA is carried out in an insect cell line to prepare the recombinant baculovirus encoding the selected recombinant protein instead of polyhedrin; 3) isolation and purification of the recombinant baculovirus; 4) preparation of recombinant protein in the cell line or insect larvae using recombinant baculovirus. Most significant disadvantage of this biotechnology is the use of an expensive and labor-intensive cell line both to prepare recombinant baculoviruses and final protein products.

Therefore, the goal of our research was to prepare recombinant baculovirus and recombinant β-galactosidase of *Escherichia coli* based on it directly in silkworm larvae. Recombinant plasmid DNA, pBL2, which contains the lacZ gene promoted by the NPV polyhedrin gene of *B. mori*, was constructed for this earlier [2]. The lacZ gene was cloned into plasmid vector of transfer pBK 273 in order to prepare recombinant plasmid pBL2 DNA [3]. This plasmid contains the ascending and descending sequence of the *B. mori* NPV polyhedrin gene, which is a mediator of homologic recombination of the expressed gene with the genome of wild *B. mori* NPV.

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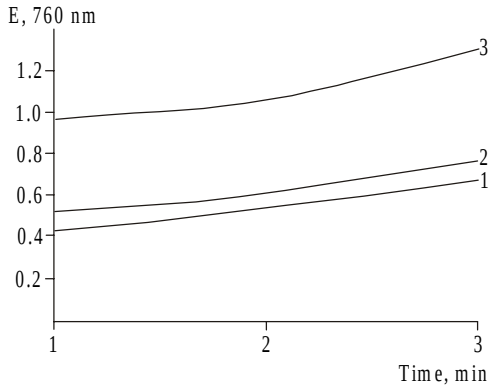

Fig. 1. Optical extinction of protein extracts from *B. mori* larvae injected with recombinant baculovirus incubated with X-gal: control (protein extract from noninjected larvae) (1), protein extract from larvae injected with a mixture of wild *B. mori* DNA NPV and plasmid DNA pBL2 (2), protein extract from larvae injected with *B. mori* recombinant NPV DNA containing the lacZ gene.

The gene lacZ was isolated from plasmid pYI356 using hydrolysis by restrictases KpnI and StuI. The vector plasmid pBK 273 was cleaved by restrictase EcoRI. The "sticky" ends were inserted by a fragment of Klenov DNA-polymerase I of *E. coli* and hydrolyzed by KpnI restrictase. The KpnI—StuI fragment of plasmid pYI356, which contains the lacZ *E. coli* gene, was cloned through these sites into the transfer plasmid. The resulting recombinant plasmid, designated pBL2, has the lacZ gene located immediately after the polyhedrin promoter gene.

NPV DNA of wild *B. mori* was mixed with pBL2 recombinant plasmid DNA containing the *E. coli* lacZ gene. The resulting mixture was injected into fifth-stage *B. mori* larve. Replication of the genetically modified baculovirus that contains the *E. coli* lacZ gene instead of the polyhedrin gene occurs owing to homologic recombination *in vivo* of the aforementioned DNA and replication of wild NPV in the larvae. The larvae were homogenized in 72 h after injection. Total virus DNA was isolated from the homogenate. We developed a modified method to separate recombinant baculovirus DNA and wild *B. mori* NPV DNA that includes DNA—DNA hybridization with subsequent elution of recombinant baculovirus DNA [4].

The product of recombinant baculovirus expression is easily identified. The presence of β-galactosidase in *B. mori* larvae was determined using the appearance of a blue color in the presence of a chromogenic indicator such as 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The blue color was visually observed practically immediately as the chromogenic substrate was introduced upon injection with an X-gal solution of *B. mori* larvae infected beforehand with recombinant baculovirus that produce β-galactosidase.

Use of purified recombinant baculovirus DNA increased significantly the level of *E. coli* recombinant β-galactosidase expression compared with the expression level after the use of a mixture of wild *B. mori* NPV DNA and recombinant plasmid pBL2 DNA (Fig. 1).

Thus, we developed a method for preparing recombinant baculovirus in *B. mori* larvae (silkworm) and avoided cultivation and the use of a cell line, which is very difficult and requires expensive equipment and reagents. This significantly reduced the cost of the recombinant protein without loss its biological properties.

EXPERIMENTAL

Enzymes from Stratagene (USA) and reagents from Sigma (USA) were used.

***E. Coli* Cell Cultivation.** A strain of *E. coli* containing pBL2 plasmid with cDNA lacZ gene under the promoter of the polyhedrin gene and an ampicillin-resistant gene was cultivated in 1×YT medium with ampicillin (30 μg/mL) at 37°C [5].

Plasmid DNA was isolated by alkaline lysis with subsequent deproteinization by the mixture of phenol—$CHCl_3$ [6].

Isolation of Wild *B. Mori* NPV DNA. A suspension (8 mL) of polyhedras isolated from larvae infected by NPV was treated with NaOH (2 M, 1 mL) and sodium dodecylsulfate (SDS, 1 mL, 10%), incubated for 2 h at 37°C, and treated with a freshly prepared soluiton (50 mL) of the following composition: SDS (0.6%), EDTA (10 mM), tris-HCl (10 mM), pH 7.5, mercaptoethanol (1 mM). The solution was added carefully along the wall with slow rocking of the flask. The resulting liquid was placed in a refrigerator (4°C) for 3 d and centrifuged at 10,000 rpm for 30 min. The supernatant was collected, treated with proteinase K or pronase until the final concentration reached 200 μg/mL and triton X-100 or SDS until the final concentration was 1%, and incubated for 2 h at 37°C. The proteins were extracted by an equal volume of phenol—$CHCl_3$ and twice with an equal volume of $CHCl_3$. Sodium chloride was added until the final concentration was 0.1 M. Three volumes of cold ethanol and 2 h at -20°C produced a precipitate that was centrifuged at 10,000 rpm for 30 min. The supernatant was poured off. The solid was dried. Virus DNA was dissolved in the required amount of distilled water.

All procedures were performed at 0-4°C. The isolated *B. mori* NPV DNA was monitored using electrophoresis in 0.7% agarose gel [6].

Transfection of Fifth-Stage *B. Mori* Larvae by a Mixture of Wild *B. Mori* NPV DNA and pBL2 Plasmid DNA. Wild *B. mori* NPV DNA was mixed with recombinant pBL2 plasmid DNA at 1:2 molar ratio. The resulting mixture, which contains ~1.5 μg/μL DNA, was injected into fifth-stage *B. mori* larvae.

Isolation of Total Baculovirus DNA. Larvae were homogenized in 72 h after injection. The homogenate was centrifuged. Supernatant was collected. Total baculovirus DNA was isolated by the same method as wild *B. mori* NPV DNA.

Isolation of Recombinant *B. Mori* NPV DNA Containing LacZ Gene. DNA—DNA hybridization with subsequent elution was used to isolate recombinant baculovirus DNA and wild *B. mori* NPV DNA. Recombinant pBL2 plasmid DNA containing *E. coli* lacZ gene was dissolved in distilled water until the concentration was 500 μg/mL, heated at 95°C for 15 min, quickly cooled, treated with an equal volume of NaOH (1 M), and incubated for 20 min at room temperature. A sample of the DNA was neutralized with 0.5 the volume of buffer containing NaCl (1M), sodium citrate (0.3 M), tris-HCl (0.5 M), pH 8.0, and HCl (1 M); stirred well; and quickly cooled. A nitrocellulose filter with pBL2 DNA (20 μg) placed on it was first dried in a stream of air and then heated at 80°C for 2 h. Thus, a nitrocellulose filter with an immobilized probe for DNA—DNA hybridization on it was prepared.

Filters were placed in a sterile Eppendorf tube (1.5 mL). Total DNA dissolved in distilled water (100 μL) was treated with hybridization solution of composition deionized formamide (65 μL), 1,4-piperazine-diethanesulfonic acid (PIPES, 10 μL, 200 mM, pH 6.4), SDS (20 μL, 10%), NaCl (10 μL, 5 M), and tRNA from calf liver (5 μL, 100 μg/mL). The hybridization solution was heated at 95°C for 15 min, added to the filters, and incubated at 50°C for 3 h.

Recombinant baculovirus DNA bound to the probe after DNA—DNA hybridization was eluted from the filter. The filter was treated with tRNA from calf liver (100 μg/mL) calculated for 300 μL per 0.5 cm^2 of filter surface. The tube was heated at 100°C for 1 min and immediately cooled in a dry-ice—ethanol bath. The sample was frozen on ice. The filter was removed. Eluted recombinant baculovirus DNA containing *E. coli* lacZ cDNA gene was deproteinized by an equal volume of phenol—$CHCl_3$ (1:1) mixture. DNA was precipitated by a three-fold excess of ethanol. The tube was placed in a dry-ice—ethanol bath for 20 min. Genetically modified baculovirus DNA containing *E. coli* lacZ cDNA gene was collected by centrifugation.

Identification of β-Galactosidase in Protein Extracts from *B. Mori* Larvae Homogenate. *B. mori* larvae were injected with a mixture of wild *B. mori* NPV DNA and pBL2 plasmid DNA and with purified recombinant baculovirus DNA and homogenized in 72 h. The homogenate was treated with tris-HCl (250 mM, pH 7.8) and ultrasound in ice. The lysate was clarified by centrifugation. The supernatant was collected. The protein concentration in the samples was estimated by the Lowry method [7].

A solution (1500 μL) of each sample was treated with X-gal (20 mg/mL) in DMF (50 μL) and held at 37°C. The absorbance was recorded on a spectrophotometer at 760 nm after 1, 2, and 3 h of incubation.

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