Vaccine xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Production of highly immunogenic virus-like particles of bovine papillomavirus type 6 in silkworm pupae

Satoko Watanabe [a,*,1], Tetsuya Iizuka [b], Shinichi Hatama [a], Toru Kanno [c], Masaji Mase [a], Tomoyuki Shibahara [a,d,1]

[a] National Institute of Animal Health, NARO, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan
[b] Institute of Agrobiological Science, NARO, 2-1 Ohwashi, Tsukuba, Ibaraki 305-8634, Japan
[c] National Institute of Animal Health, NARO, Hokkaido Research Station, 4 Hitsujigaoka, Toyohira, Sapporo, Hokkaido 062-0045, Japan
[d] Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-58 Rinku-oraikita, Izumisano, Osaka 598-8531, Japan

ARTICLE INFO

Article history:
Received 31 May 2017
Received in revised form 19 August 2017
Accepted 30 August 2017
Available online xxxx

Keywords:
Silkworm
Bovine papilloma virus type 6
Teat papillomatosis
Vaccine
Virus-like particle

ABSTRACT

Bovine papillomaviruses (BPVs) are the causative agent of bovine teat papillomatosis, which can lead to severe economic losses in dairy cattle. Among the 14 identified BPV genotypes, BPV type 6 (BPV6) is the most frequently detected in teat papilloma lesions, and is therefore thought to play a major role in teat papillomatosis. To develop an effective vaccine against BPV6 infection, we produced virus-like particles of BPV6 (BPV6-VLP) in silkworm (*Bombyx mori*) pupae and purified these by heparin affinity chromatography using a single column. About 0.7 mg purified BPV6-VLP was obtained from one pupa. BPV6-VLP-immunized mice produced a specific IgG to BPV6 that recognized BPV6 antigen with high sensitivity in an immunohistochemical analysis. Thus, silkworm pupae are a useful bioreactor for the production of BPV6-VLP, which can potentially be used as a vaccine for bovine teat papillomatosis.

1. Introduction

Papillomaviruses (PVs) infect epithelia in humans and animals and cause benign hyperproliferative lesions—commonly known as warts or papillomas—that can occasionally progress to squamous cell cancer. Bovine papillomatosis caused by bovine papillomavirus (BPV) infection afflicts dairy cattle herds worldwide [1]; the associated papilloma lesions in teats can lead to tissue deformation, making it difficult to milk the cows. Poor milking can in turn predispose these animals to mastitis and distortion of the milk ducts. Thus, teat papillomatosis can lead to considerable economic losses for dairy farmers.

To date, 14 BPV genotypes have been identified through genome sequencing and phylogenetic analyses [2,3]. Of these, BPV type 6 (BPV6) is the most frequently detected in teat papillomatosis lesions and is therefore considered as an important target for the management of teat papillomatosis [4,5], for which there are currently no effective commercial prophylactic or therapeutic vaccines. Since PVs cannot be cultured *in vitro* for the production of virions or viral proteins that can serve as a source of antigens, live attenuated or inactivated vaccines cannot be produced. Thus, PV vaccines must be developed by genetic engineering.

Virus-like particles (VLPs) exhibit the morphological and immunological features of native virions, but are not infectious and do not cause disease owing to the absence of a genome [6–8]. VLPs composed of the major viral capsid protein L1 can be used to develop vaccines directed against PVs. VLPs of BPV types 1, 2, and 4 have been generated from baculovirus-insect-, yeast-, and plant-based expression systems [9–11]. However, for VLPs to be used in this manner, a safe and practical large-scale expression system is required. Moreover, in the case of veterinary vaccines, the cost of a single dose must be weighed against the value of the vaccinated animal. The silkworm (*Bombyx mori*) is economically important for the production of silk and is also used as a bioreactor for the production of heterologous recombinant proteins, as it offers distinct advantages such as high protein yield and posttranslational modifications similar to those in vertebrates [12–14]. Silkworms can be considered as small, independent biofactories that maintain optimal cell conditions through homeostatic mechanisms such as gas exchange, nutrient supplementation, and waste excretion. Silkworm pupae can survive at 4 °C for a long time and do not require mulberry leaves in an artificial diet during growth [14]. Given that silkworms have been domesticated through the long history of industrial silk production, they are unable to survive or

* Corresponding author.
E-mail address: satochan@affrc.go.jp (S. Watanabe).
[1] These authors equally contributed to this study.

http://dx.doi.org/10.1016/j.vaccine.2017.08.079
0264-410X/

reproduce in a natural environment, which reduces potential biohazard of using silkworms to generate recombinant proteins. Compared to other recombinant expression systems, the silkworm does not require sterile conditions or growth medium during the production process and scaling up is straightforward since a large breeding space is not required, which significantly lowers production cost. More importantly, larger amounts of recombinant proteins can be generated in silkworms than from insect and animal cell cultures. For example, the expression level of Japanese encephalitis virus VLPs was about 15–25 fold higher in silkworm pupae than in cultured insect cells [15], and mouse interleukin-3 activity was about 20– to 10,000–fold higher in the hemolymph of silkworm larvae than in culture supernatants of BmN and COS7 cells, respectively [16]. The high expression level of recombinant proteins generated in silkworm can lower production costs. Silkworm pupae have also been used to produce a wide variety of VLPs including canine parvovirus capsid protein, foot-and-mouth disease virus capsid protein, and the highly pathogenic avian influenza virus hemagglutinin [17–19].

In this study, we produced the major BPV6 capsid protein L1 in silkworm pupae to investigate its potential as a vaccine antigen. L1 expression, the correct assembly of BPV6-VLPs in vivo, and the immunogenicity of purified BPV6-VLPs in mice were evaluated.

2. Materials and methods

2.1. Recombinant baculovirus production and recombinant protein expression

The L1 gene of BPV6 (BPV6-L1) was amplified from BPV6 DNA isolated from a teat papilloma lesion and cloned into the *Bam*HI site of the baculovirus transfer vector pAcYM1 [20]. The recombinant transfer vector and the DNA of the hybrid baculovirus, hybrid nuclear polyhedrosis virus (HyNPV)—which can infect all hosts of *Autographa californica* NPVand *B. mori* NPV [21]—were co-transfected into *Spodoptera frugiperda*-derived Sf21AE cells cultured in SF900 II medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum. BPV6-L1 containing the recombinant baculovirus HyBPV6-L1 was isolated as previously described [22].

To obtain recombinant protein, silkworm pupae shortly after larval-pupal ecdysis were injected with 10 μl of HyBPV6-L1 containing 1×10^7 plaque forming units/ml and bred at 25 °C. Six days later, infected pupae were collected and stored at −80 °C.

2.2. Immunohistochemical analysis of BPV expression in pupae

Baculovirus-infected pupae were fixed by immersion in 10% phosphate-buffered formalin and embedded in paraffin. After deparaffinization of the tissue sections, endogenous peroxidase activity was blocked by treatment with methanol and 3% H_2O_2, followed by incubation with a rabbit polyclonal antibody against BPV type 1 (BPV1) (Dako, Carpinteria, CA, USA) (1:256 dilution). After 1 h, the immunocomplex was detected by the streptavidin–biotin–alkaline phosphatase method using the Histofine SAB-PO kit (Nichirei, Tokyo, Japan). Sections were lightly counterstained with Mayer's hematoxylin and examined by light microscopy.

2.3. Purification of recombinant protein

Recombinant BPV6-L1 was purified from silkworm pupae as previously described [23], with minor modification. A single frozen pupa was ground in liquid nitrogen and sonicated in 5 ml buffer A composed of 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, and 0.33 M NaCl (pH 7.0) and containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Sonication (three cycles of six pulses for 30 s) was performed using an Ultra S Homogenizer VP-15S (Titec, Tokyo, Japan). The homogenate was centrifuged at 3000 rpm for 20 min; the supernatant was further centrifuged at 11,000 rpm for 20 min and the supernatant from the second centrifugation was sequentially filtered with different pore sizes (0.8, 0.45, and 0.2 μm). After filtration, 500 μl of filtrate was passed through a HiTrap Heparin HP column (0.7 × 2.5 cm; GE Healthcare, Milwaukee, WI, USA) equilibrated with buffer A at 1 ml/min. The column was washed with 10 column volumes of 10% buffer B composed of 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, and 2 M NaCl (pH 7.0) and was eluted with a linear gradient from 10% to 100% buffer B for 10 min. The absorbance of the eluted proteins was measured at 280 nm. Eluted fractions were collected and dialyzed against phosphate-buffered saline (PBS) for further analysis.

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

A 5-μl volume of each sample was separated by SDS–PAGE under reducing conditions [24]. The gel was stained with Coomassie Brilliant Blue G-250 to visualize protein bands.

2.5. Transmission electron microscopy (TEM) analysis

The recombinant BPV6-L1 protein fractions were adsorbed on a 400-mesh collodion membrane-coated copper EM grid (Nisshin EM, Tokyo, Japan) for 5 min and stained with 2% uranyl acetate for 30 s. The morphology of recombinant BPV6-L1 was examined by TEM (H-7500; Hitachi, Tokyo, Japan). The diameter of 100 particles was measured using AMTV 542 software (Advanced Microscopy Techniques, Danvers, MA, USA), and the average diameter is presented as mean ± standard deviation.

2.6. Determination of protein concentration

Protein concentrations were determined using the Detergent-compatible protein assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard.

2.7. Mice immunization and sample collection

Six-week-old female BALB/c mice (Japan SLC Co., Hamamatsu, Japan) were immunized by intramuscular injection with 20 μg BPV6-VLP (5 mice) in 50 μl PBS without adjuvant and boosted 14 days after the first immunization. As a negative control, 50 μl PBS 3 mice) was injected. Blood was collected 7, 14, 21, 28, 35, 42, and 49 days after the first immunization, and sera were recovered by centrifugation and stored at −80 °C. Animal experiments were carried out according to regulations and guidelines of the Animal Ethics Committee of the National Institute of Animal Health (approval number 16-053).

2.8. Enzyme-linked immunosorbent assay (ELISA)

Maxisoap 96-well plates (Corning Inc., Corning, NY, USA) were coated with BPV6-VLP overnight at 4 °C. After blocking with 300 μl of BlockAce solution (Dainippon Pharmaceutical, Suita, Japan), serum samples diluted 100 fold were added to the wells followed by incubation for 1 h at room temperature. Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (1:150,000 dilution) or HRP-conjugated rabbit anti-mouse IgM (1:100,000 dilution) was added to the wells for 1 h at room temperature. The colorimetric reaction was carried out by incubating the plates with 100 μl 3,3′,5,5′-tetramethylbenzidine solution (KPL, Gaithersburg, MD, USA) for 5 min at room temperature. After terminating

Please cite this article in press as: Watanabe S et al. Production of highly immunogenic virus-like particles of bovine papillomavirus type 6 in silkworm pupae. Vaccine (2017), http://dx.doi.org/10.1016/j.vaccine.2017.08.079