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BRIEF COMMUNICATION

Optimum storage conditions for product of transiently expressed epitopes of *Human papillomavirus* using *Potato virus X*-based vector

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Abstract

We describe the optimized storage conditions of recombinant *Potato virus A* coat protein (ACP) carrying two different epitopes from *Human papillomavirus* type 16 (HPV-16). Epitope derived from minor capsid protein L2 was expressed as N-terminal fusion with ACP while an epitope derived from E7 oncoprotein was fused to its C-termini. The construct was cloned into *Potato X potexvirus* (PVX) based vector and transiently expressed in plants using *Agrobacterium tumefaciens* mediated inoculation. The effect of storage conditions on the serological activity of L2ACPE7 was studied by ELISA using IgG anti PVX, PVA and L2. Purified L2ACPE7 stored freeze-dried (at -20 °C), frozen at various temperatures (-20 °C, -70 °C) and at +4 °C were tested. Purified L2ACPE7 was most stable as lyophilized material stored at -20 °C. Our study demonstrates suitable way for the storage of plant material containing foreign viral epitopes for the purposes of edible vaccination.

Additional key words: edible vaccination, heterologous proteins, *Nicotiana benthamiana*, serological activity.

A phytovirus-based vector is a useful tool for efficient expression of target protein in plants. It can provide production of target protein up to 40 % of total soluble protein of the cell (Gleba *et al*. 2004). Plant expression systems have a significant advantage compared to other methods of recombinant protein production since plants are much cheaper and easier in cultivation than cell cultures. Plant viral vectors are being successfully applied for production of pharmaceuticals (Canizares *et al.* 2006).

Human papillomavirus (HPV) infection is the causative agent of cervical cancer. HPV16 is the most prevalent type of HPV associated with cervical cancer, detected in about 50 % and it is therefore the most important target for development of both prophylactic and therapeutic anti-HPV vaccines. Preventive immunization is based on the structural L1 and L2 proteins of HPV and should result in induction of neutralizing antibodies. Therapeutic vaccination against HPV is aimed at eliciting cellular immune responses to viral E7 and/or E6 oncoproteins that are the only viral proteins continually expressed in cells of cervical cancer. As a model protein for fusion and evaluation of expression of HPV16 epitopes in plants, we chose *Potato virus A* coat protein (ACP) because of the efficient and reliable way of its detection by means of our own polyclonal antibodies. Furthermore, ACP is an attractive epitope carrier suitable for production of vaccines in plants because of its possibility to form non-replicative self-assembling virus like particles based on its coat protein. Such virus-like particles can present foreign epitopes on their surface (Jagadish *et al*. 1996). These recombinant virus-like particles can directly serve as an immunizing agent.

The sequence of the 17aa epitope from E7 protein (QAEPDRAHYNIVTFCCK) (Tindle *et al*. 1991) was

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Abbreviations: ACP - *Potato virus A* coat protein; HPV - *Human papillomavirus*; PAbs - polyclonal antibodies; pGR106 - binary vector; PVA - *Potato virus A*; PVX - *Potato virus X*.

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fused with the 3´-terminus of the coding sequence of ACP isolate LI using two sequential polymerase chain reactions (PCR) with primers having long 5'-overhang. At the same time the 5´-terminus was extended to accommodate the sequence coding for the 14 aa epitope from L2 protein (LVEETSFIDAGAPG) (Pokorna *et al*. 2005). The PCR product was cloned to pUC57T/A (*Fermentas*, Burlington, Canada) using 3'-A overhangs generated by Taq polymerase. After the correct sequence was confirmed by sequencing the fusion gene was subcloned into PVX expression vector pGR106 (PVX; kindly provided by Dr. D.C. Baulcombe (The Sainsbury Laboratory, Norwich, GB) using NotI/SalI restriction sites.

The ACP gene was prepared by reverse transcription polymerase chain reaction (RT PCR). The primers for amplification of coat protein coding region were designed according to the sequence of isolate PVA LI, Acc. No. Z21670 (Puurand *et al.* 1994).

The L2ACPE7 construct in pGR106 was electroporated into *Agrobacterium tumefaciens* (GV3101) and bacteria containing the plasmid were selected on LB-agar plates containing 50 μg cm^{-3} kanamycin at 28 °C. Bacterial suspension for agroinfection of plants was prepared in LB-medium containing 50 μg cm^{-3} kanamycin at 28 °C. First and second true leaves of greenhouse grown four-leaf stage *Nicotiana benthamiana* (grown under controlled conditions (20-25 °C over day, 15-20 °C over night, 16h-light/8h-dark cycle) were injected with the suspension of *Agrobacterium tumefaciens* containing L2ACPE7 (Čeřovská *et al.* 2004) using a syringe without a needle. Inoculated leaves were harvested 8 days post inoculation (dpi) and stored in -70 °C until analyzed. The first and second true leaves of experimental *N. benthamiana* plants in their four-leaf stage were mechanically inoculated by rubbing the carborundum-dusted first and second true leaves with the extract of the agroinfected leaves (dillution 1:10 in 0.057 M KH_2PO_4). Four top leaves of infected experimental plants were harvested 14 dpi and analysed.

The purification of expressed L2ACPE7 was carried out according to Čeřovská *et al*. (1991). The serological activity of purified L2ACPE7 was determined by the indirect ELISA. For the plate-trapped antigen form of indirect ELISA (Mowat 1985), three steps were considered. In step 1 the wells contained purified L2ACPE7 (10 μg cm^{-3}) in a coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, pH 9.6). In step 2 this solution was replaced by PAbs diluted in 2 % PVP and 0.2 % ovalbumin in PBS+T (37.0 mM NaCl, 8.0 mM Na_2HPO_4, 15.0 mM KH_2PO_4, 3.0 mM NaN_3, 2.7 mM KCl, 0.05 % *Tween 20*, pH 7.4). We used rabbit polyclonal IgG antibodies against PVA (5 μg cm^{-3}, Table 1), PVX (1:1000, Table 2; both PVA and PVX antibodies kindly provided by Dr. P. Dědič, Potato Research Institute, Havlíčkův Brod, Czech Republic; Table 2) and against L2 (5 μg cm^{-3}, kindly provided by Dr. M. Sapp, Center for Molecular and Tumor Virology, Shreveport, USA; Table 3). In step 3 linked PAbs were detected by polyclonal goat anti-rabbit antibody IgG-alkaline phosphatase conjugate (1:10 000 in 2 % PVP and 0.2 % ovalbumin in PBS+T; *Sigma*, Milwaukee, USA) and the substrate of the alkaline phosphatase (*p*-nitrophenyl phosphate; *Sigma*). The values in the tables are means from 3 experiments performed in duplicates.

In long-term experiments we tested the purificate serological activity in freeze-dried samples and samples stored frozen at -20, -70 and +4 °C for 1, 3, 6, 9 and 12 months after purification.

Table 1. Effect of storage conditions on serological activity of the purified L2ACPE7. Activity of the construct was detected by rabbit IgG against PVA. Means ± SE, $n = 6$.

Storage [months]	Serological activity of purified construct [%] lyophilized	+4 °C	-70 °C	-20 °C
0	100 ± 4.2	100 ± 5.1	100 ± 3.3	100 ± 4.1
1	76 ± 2.7	56 ± 4.4	54 ± 5.8	56 ± 4.0
3	65 ± 3.1	37 ± 2.0	42 ± 2.6	42 ± 2.6
6	34 ± 2.8	26 ± 2.9	38 ± 2.7	22 ± 1.8
9	28 ± 1.3	20 ± 2.5	31 ± 1.7	20 ± 1.8
12	21 ± 1.5	16 ± 2.6	12 ± 1.9	12 ± 2.0

Table 2. Effect of storage conditions on serological activity of the purified L2ACPE7. Activity of the construct was detected by rabbit IgG against PVX. Means ± SE, $n = 6$.

Storage [months]	Serological activity of purified construct [%] lyophilized	+4 °C	-70 °C	-20 °C
0	100 ± 4.4	100 ± 4.7	100 ± 4.2	100 ± 4.7
1	96 ± 3.8	64 ± 6.2	85 ± 2.8	68 ± 5.0
3	87 ± 2.4	50 ± 4.7	69 ± 3.1	17 ± 3.5
6	77 ± 2.8	16 ± 2.6	69 ± 3.7	16 ± 2.6
9	42 ± 4.2	23 ± 3.4	42 ± 2.4	9 ± 2.7
12	40 ± 3.4	21 ± 3.3	35 ± 2.2	9 ± 3.1

Table 3. Effect of storage conditions on serological activity of the purified L2ACPE7. Activity of the construct was detected by rabbit IgG against L2 HPV. Means ± SE, $n = 6$

Storage [months]	Serological activity of purified construct [%] lyophilized	+4 °C	-70 °C	-20 °C
0	100 ± 3.2	100 ± 4.4	100 ± 3.1	100 ± 2.7
1	69 ± 2.7	20 ± 2.7	25 ± 4.5	0.2 ± 4.8
3	20 ± 1.9	0.4 ± 2.4	-0.8 ± 3.4	-0.5 ± 3.6
6	0.8 ± 2.3	0.1 ± 3.1	0.2 ± 2.9	0.4 ± 3.9
9	-0.2 ± 1.7	-0.7 ± 1.1	-0.3 ± 2.7	0.4 ± 2.4
12	-0.5 ± 2.1	0.4 ± 2.3	0.7 ± 1.3	0.8 ± 1.8

The serological activity of purified L2ACPE7 detected by PAbs against PVA (Table 1) decreased after 6 months to 34 - 38 % in samples stored frozen at -70°C and freeze-dried, to 26 % in the sample stored at +4 °C and even less activity was detected in purificate stored at -20 °C. After 12 months most active was freeze-dried purificate (21 %), but activities detected in the samples stored at -70, -20 and at +4 °C were lower.

The serological activity detected by PAbs against PVX (Table 2) after 1 month decreased only slightly in the freeze-dried purificate (to 96 %), after 6 months it was 77 % in the freeze-dried purificate, 69 % in the sample stored frozen at -70 °C and only 16 % in the sample stored at +4 °C. After 12 months the highest activities were again detected in freeze-dried purificate (40 %) and also in purificate stored at -70 °C (35 %).

The serological activity detected by PAb against L2 (Table 3) after 1 month was not detected in purificate stored at -20°C, after 3 months it was detected only in lyophilized purificate (20 %).

According to our opinion the lower activity of purificate stored at -20 °C than at +4 °C is possible to explain by lower stability of virus-like particles during slow freezing procedure without cryoprotectants which help to stabilize proteins by preventing the formation of ice crystals that destroy protein structure.

The aim of our work was to optimize the storage conditions for isolated expressed protein of L2ACPE7 construct, which could be used for immunological studies for development of vaccine purposes. Comparing the serological activities obtained with three antibodies raised against different parts of our construct we determined that the best method for a long-term storage is freeze-drying of the purificated L2ACPE7. Furthermore, the advantage of this storage procedure might be concentration of expressed protein in plant tissue and direct use of lyophilized tissue for tablets preparation. In general, this procedures could be used for efficient storage of recombinant antigens in plants and could serve also as a good model system for other recombinant antigens prepared in plants for edible vaccination.

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