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RESEARCH ARTICLE

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Baculoviral infection reduces the expression of four allergen proteins of silkworm pupa

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Abstract

Silkworm (*Bombyx mori*) larvae are widely used to express exogenous proteins. Moreover, some silkworm pupal proteins can be used as drug-loading materials for selfexpressed oral tolerance drugs. However, several proteins expressed in silkworm pupae cause severe allergic reactions in humans and animals. Interestingly, some baculovirus vectors have been shown to alter the host gene and its expression in insect cells, but this has not been confirmed in silkworm. Here, we analyzed the effects of infection with an empty B. mori baculovirus (BmNPV) vector on silkworm pupal protein expression. Using a proteomics approach, the allergens thiol peroxiredoxin (Jafrac1), 27-kDa glycoprotein (p27k), arginine kinase, and paramyosin as well as 32 additional differentially expressed proteins were identified. Down-regulation of the messenger RNA expression of the four known allergens was observed after BmNPV infection; subsequent changes in protein expression were confirmed by the western blot analysis using polyclonal antibodies prepared with recombinant proteins of the four allergens. Collectively, these data indicate that the four known allergens of silkworm pupae can be reduced by infection ith an empty BmNPV vector to increase the safety of silkworm pupa-based exogenous protein expression and drug delivery of oral pharmaceuticals. In addition, the four recombinant allergen proteins may contribute to the diagnosis of allergic diseases of silkworm pupa.

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KEYWORDS
allergen, baculovirus vector, bioreactor, silkworm pupa

1 | INTRODUCTION

Silkworm (*Bombyx mori*) is widely used as a eukaryotic expression system and as a bioreactor (Maeda et al., 1985). These bioreactors not only effectively perform posttranslational modifications (Herrington et al., 1992; Kato, Kajikawa, Maenaka, & Park, 2010; Lu, Farrell, Johnson, & Iatrou, 1997) but also tend to incorporate the native antigenicity and enzyme activity into the expression product (Reis, Blum, von Specht, Domdey, & Collins, 1992). Notably, this system does not involve *Escherichia coli* endotoxins, the expression product glycosylation, or other factors and processes that have been shown to limit protein expression activity (Farrell et al., 1998). The *B. mori* baculovirus (BmNPV) is an ideal expression vector for this bioreactor. The major advantages of this expression vector are that the closed-loop double-stranded DNA of BmNPV is easily genetically modified and it contains a strong promoter for recombinant protein expression in insect cells (J. Zhang, Kalogerakis, Behie, & Iatrou, 1993). In addition, a nonessential polyhedrin gene locus can be used to insert foreign genes (Johnson, Schmiel, Iatrou, & Gedamu, 1993). Furthermore, because of its host specificity, BmNPV does not induce any pathogenic infection to humans or livestock (Carbonell & Miller, 1987; Herrington et al., 1992) and it is easily inactivated in the natural environment after recombination (Bianchi, Joosten, Vlak, & van der Werf, 2000; Inceoglu et al., 2001). These characteristics make BmNPV-mediated protein expression in *B. mori* both simple and effective.

Silkworm pupae are rich in lipoproteins and can encapsulate self-expressed exogenous proteins to form microcapsules. These lipid-soluble microcapsules have been shown to enter intestinal epithelium cells via pinocytosis and promote absorption (W. Zhang et al., 2009). Silkworm pupal liposomes can also protect drugs and reduce the risk of their degradation by intestinal enzymes (Higashihashi et al., 1991; Li et al., 2011; Shi, Qin, Zhu, & Zhu, 1996), enabling slow release of a drug into the body and a prolonged action time (Gong, Jin, & Zhang, 2005). Therefore, the physiological and pharmacological activities of these silkworm pupal microcapsules make them ideal drug carriers for oral tolerance drugs (Shirai, Fujii, Doira, & Iwamoto, 2000; Shrivastava & Ghosh, 2003; Yamamoto, Watabe, Kageyama, & Takahashi, 1999). Indeed, their use in biomedical research has been highlighted by the successful delivery of recombinant human granulocyte-macrophage colony stimulating factor (W. Zhang et al., 2009) and the cholera toxin B subunit (Gong et al., 2005).

However, many silkworm pupal proteins including arginine kinase (Binder et al., 2001), thiol peroxiredoxin (H. Wang, Hu, & Liang, 2016), and p27k, a putative cuticle protein (Jeong et al., 2016) can cause allergic reactions in some patients. In India, silk-related occupations rank the highest with regard to occupational asthma development and incidence (Harindranath, Prakash, & Subba Rao, 1985). In Japan, the silkworm moth has been identified as a trigger of allergic rhinitis (Suzuki et al., 1995). In southern China, nearly 50% of allergy patients are positive for serum immunoglobulin E (IgE) against silkworm pupae (Araujo, Rosário Filho, & Riedi, 2014; Sun, Zheng, Wei, Huang, & Zeng, 2014; S. Wang et al., 2014). The silkworm pupa allergen content in most foods that are typically considered as allergenic exceeds 1% of the total protein (Ermel, Kock, Griffey, Reinhart, & Frick, 1997). Allergic responses in animals were shown to be consistent with those in humans (Helm & Burks, 2002). As various silkworm pupal proteins appear to be severe allergens (Larsson & Backlund, 2009; Z. Liu et al., 2009), the use of silkworm microcapsules as drug delivery tools is restricted. Thus, blocking or reducing the allergic anaphylactic reaction to these silkworm proteins is essential.

Previous studies have found that viruses have their own protective mechanisms in the process of their interaction with host cells. Self-propagation, exploitation of posttranslational modification, and control of nonhomologous end joining through host cells are the major tactics used by viruses to collapse their host's genome. In addition, the common approaches used by viruses to maximize proliferation include ubiquitin targeting and induced degradation of host proteins. These approaches lead to the destruction of key proteins or the expression of

important genes, causing a rapid reduction in the resistance of the host to the virus (Isaacson & Ploegh, 2009). However, in the silkworm bioreactor with BmNPV as a vector, the regulation of allergen proteins has hardly been reported.

In the present study, we further analyzed the regulation of allergen proteins by the empty BmNPV vector after infection with silkworm pupa. We used various proteomic, genomic, and molecular biology techniques to determine the effects of the empty BmNPV vector on silkworm allergens.

2 | MATERIALS AND METHODS

2.1 | Silkworm pupae and viral vector preparation

Recombinant baculovirus vector culture fluid was provided by the Biotechnology Institute of the Chinese Academy of Agricultural Sciences (Beijing, China) and stored at 4°C until use. The experimental silkworm pupae were obtained from the Huzhou Academy of Agricultural Sciences (Huzhou, China). Healthy silkworm pupae were selected and disinfected with 0.3% calcium hypochlorite. After drying, the silkworm pupae were inoculated with empty recombinant BmNPV vector at 105 PFU/head (5 μl/head) and incubated at 25–26°C and 65–70% relative humidity for 5 days. The infected silkworm pupae were then collected and stored at −20°C. Untreated silkworm pupae were used as a control group.

2.2 | Protein preparation

Extraction of infected and uninfected silkworm pupal proteins was performed using trichloroacetic acid acetone precipitation. See Supporting Information Materials for details.

2.3 | Two-dimensional gel electrophoresis (2-DE)

Details of 2-DE are provided in Supporting Information Materials.

2.4 | Mass spectrometry

To identify differentially expressed proteins in the 2-DE assay, we extracted colloidal particles from the gel and identified them by mass spectrometry. See Supporting Information Materials for details.

2.5 | RNA isolation and cDNA synthesis

Total silkworm pupal RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was extracted from three silkworm pupae treated with BmNPV and three untreated silkworm pupae. The NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to quantify the RNA concentrations. The purity of all RNA samples was assessed at $A_{260/280}$ and $A_{260/230}$ absorbance ratios, and RNA integrity was confirmed by 1% agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized using a first-strand cDNA synthesis kit (Roche, Basel, Switzerland) according to the manufacturer's instructions.

2.6 | Quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

The relative messenger RNA (mRNA) expression levels of four known allergens were confirmed by RT-qPCR. The primers used in this analysis are listed in Table 1. RT-qPCR samples were prepared with an SYBR Premix Ex Taq II

TABLE 1 Primers used for RT-qPCR in this study

Primer names	Length	T_m	GC (%)	Sequences
p27k R	25	64.1	56.0	GGACGTCCGAATTGAAGACGTCCC
p27k F	28	64.7	50.0	GCTTCTGATGGTGAAACCCATGAAGAGC
Jafrac1 R	22	63.7	63.6	CTTGCGGAACTCGTCCGCCTTC
Jafrac1 F	23	63.5	63.0	GCCTCTGCAGATGACCAAACCCG
Arginine kinase R	26	65.2	53.8	GCATCCAATCGGGTGTCGAGAACTTG
Arginine kinase F	23	67.3	69.6	CGGGTGGAGACCACGAACTCGCC
Paramyosin R	25	65.1	56.0	CGAGGAACTGAACCGCACCATTGTG
Paramyosin F	27	64.8	59.3	CTCAAGCAGAGAGCGCCTTCTCTCCTC
α-Tubulin R	18	61.3	66.1	ATCAACTACCAGCCACCC
α-Tubulin F	17	62.1	69.8	CTCCCTCCTCCATACCCT

Note. RT-qPCR: quantitative reverse-transcription polymerase chain reaction; T_m: primer melting temperature.

Kit (Tli RNaseH Plus; Takara, Shiga, Japan) and quantitative fluorescence PCR kit (Bao Bio-engineering (Dalian) Co., Ltd., Dalian, China) according to the manufacturer's instructions. The reactions were carried out on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The thermal cycling profile consisted of an initial denaturation step at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. All assays were performed in triplicate. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method; α-tubulin was used as a reference gene.

2.7 | Recombinant protein expression and polyclonal antibody preparation

To verify the protein expression of the four known allergens before and after infection, we first prepared rabbit polyclonal antibodies against these proteins. The DNA fragments of Jafrac1, p27k, arginine kinase, and paramyosin were ligated into pET-28a vectors (Takara) and transformed into Rosetta cells (Solarbio, Beijing, China) for expression. Bacterial expression was induced by isopropyl-D-thiogalactopyranoside at 18°C for 20 hr. The bacteria were then harvested and resuspended in 50 mM Tris-HCl, 100 mM NaCl, pH 7.5. After sonication, these proteins were purified by affinity chromatography and their concentrations were measured. The purified recombinant proteins were diluted with an equal volume of Freund's complete adjuvant, mixed, and emulsified. Each purified protein was subcutaneously injected into two New Zealand rabbits at an initial dose of 800 μg/rabbit. After 2 weeks, we performed a second immunization using 400 μg/rabbit of protein and Freund's complete adjuvant. Additional immunizations were administered every week for 4 weeks, and the doses were increased over time. Four days after the fourth immunization, blood was collected from the heart, and serum was isolated from each sample. The antibody titer was determined by indirect enzyme-linked immunosorbent assay (ELISA).

2.8 | Western blot analysis

Western blot analysis was conducted to confirm the differential protein expression of the four allergens. In brief, equal amounts of protein from BmNPV-infected and untreated silkworm pupae were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. The proteins were transferred to nitrocellulose filter membranes, which were blocked with 5% nonfat dried milk in TBS containing 0.1% Tween 20 (TBST) for 1 hr at room temperature. The membranes were incubated with the polyclonal antibody (diluted 1:8,000) and anti-glyceraldehyde-3 phosphate dehydrogenase antibody (GAPDH, diluted 1:2,000; ABM40135; Abbkine, Wuhan, China) for 1 hr at 37°C. All antibody dilutions were prepared in TBST. The membranes were washed three times

with TBST and labeled with goat anti-rabbit IgG (bs-0295G-SA; BIOSS, Beijing, China) conjugated to horseradish peroxidase (diluted 1:5,000; bs-0295G-HRP; BIOSS) for 2 hr at 37°C. The membranes were then washed three times with PBS containing 0.1% Tween 20, followed by visualization with Western Bright (Advantsa, San Jose, CA).

3 | RESULTS

3.1 | Comparative proteomic analysis by 2-DE

The 2-DE was performed to analyze the protein expression in BmNPV-infected and untreated silkworm pupae. Gel images revealed >200 protein spots in each sample. These proteins were mainly distributed in the neutral range of isoelectric points (5–8) and had molecular weights of 20–95 kDa. Using PDquest8.0, we compared the

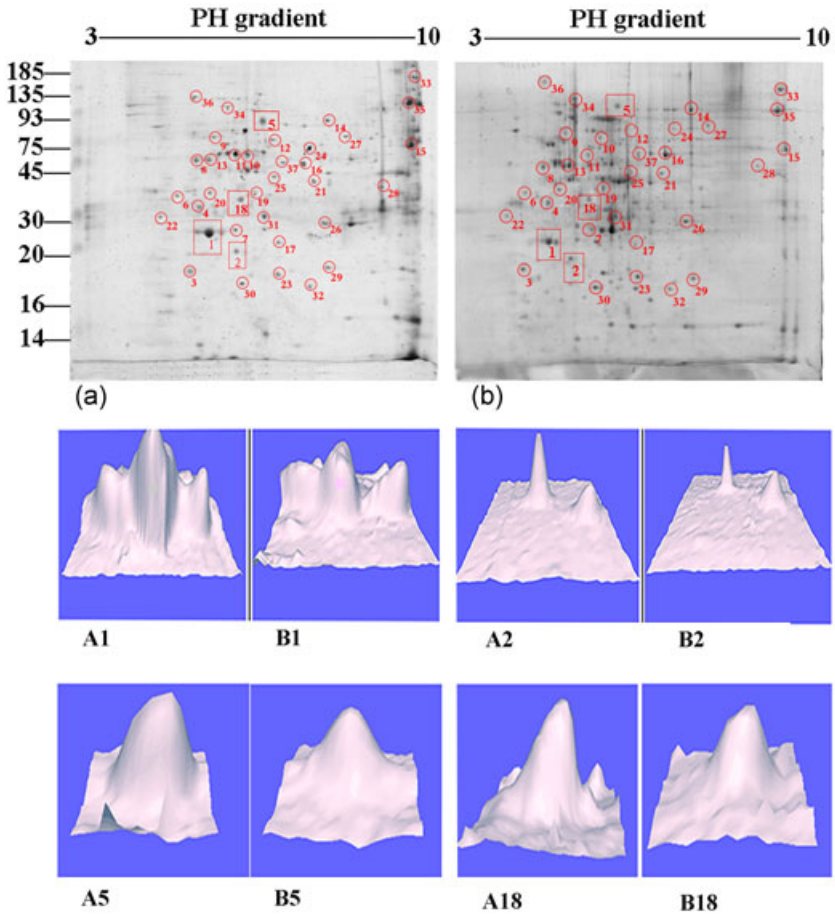


FIGURE 1 Silkworm pupa extracts were separated by two-dimensional gel electrophoresis (2-DE). (a) Image of the 2-DE gel showing the proteins expressed in untreated silkworm pupa. (b) Image of the 2-DE gel showing the proteins expressed in baculovirus (BmNPV)-infected silkworm pupa. The labels 1, 2, 5, and 18 in both images correspond to the four known allergens, respectively. A1: p27k three-dimensional (3D) view of uninfected BmNPV; B1: p27k 3D view of BmNPV empty vector infection; A2: Jafrac1 3D view of uninfected BmNPV; B2: Jafrac1 3D view of BmNPV empty vector infection; A5: paramyosin 3D view of uninfected BmNPV; B5: Paramyosin 3D view of BmNPV empty vector infection; A18: arginine kinase 3D view of uninfected BmNPV; B18: arginine kinase 3D view of BmNPV empty vector infection

three-dimensional view of infected and untreated silkworm pupae. The results showed that two proteins were significantly downregulated after viral infection (Figure 1).

3.2 | Protein identification

To identify all proteins observed by 2-DE analysis, we conducted MALDI TOF/TOF. A total of 36 protein points was successfully identified (Table 2), including four allergenic proteins as well as various immune-related proteins, such as antitrypsin isoform, vitellogenin, and serine proteinase-like protein.

3.3 | RT-qPCR

To determine the effect of BmNPV infection on the mRNA expression of the four allergens, we utilized RT-qPCR. Notably, the relative expression of the four proteins was significantly downregulated in silkworm pupae after BmNPV infection ($P < 0.05$; Figure 2), indicating that the gene expression of the four allergen proteins is inhibited by the BmNPV vector in silkworm pupae.

3.4 | Recombinant protein expression and western blot analysis

We successfully expressed the recombinant proteins of the four allergens based on SDS-PAGE analysis, showing that the molecular weights of the purified proteins were close to the expected values of 25, 22, 102, and 40 kDa, respectively (Figure 3). The concentrations of the four proteins were 1.95, 2.14, 1.39, and 2.67 mg/ml for p27k, Jafrac1, arginine kinase, and paramyosin, respectively. These purified recombinant proteins of the four allergens were used as antigens to immunize New Zealand white rabbits four times to prepare polyclonal antibodies. The antibody titers in the rabbit serum based on indirect ELISA were 1:25,600, 1:12,800, 1:25,600, and 1:51,200 for p27k, Jafrac1, arginine kinase, and paramyosin, respectively.

The polyclonal antibodies were used for the western blot analysis. Our results showed that the expression of the four allergen proteins was clearly downregulated in silkworm pupae infected with BmNPV (Figure 4). These data support our RT-qPCR and proteomic results, revealing a decrease in the expression of these genes.

4 | DISCUSSION

In the present study, we infected silkworm pupae with an empty BmNPV vector to evaluate any subsequent changes in the hypoallergenic proteins. This is the first proteomic, genomic, and molecular biology analysis of the effects of BmNPV infection on silkworm allergens.

Our results indicate that following infection with the empty BmNPV vector, a total of 36 proteins were differentially expressed in the silkworm pupae. The functions of these proteins are diverse. A previous study also detected significant changes in the protein expression following baculovirus infection. For example, Liu, Shen, Li & Wang, (2009) observed changes in RNA and protein synthesis in BmNPV-infected Bombyx mori ovary cells, which appeared to be caused by the blockage of the cell cycle in the G2/M phase. These data are consistent with the results of both Braunagel, Parr, Belyavskyi, and Summers (1998) and Ikeda and Kobayashi (1999) in studies using *Autographa californica* nucleopolyhedrovirus to infect Sf9 cells, thus altering gene and protein expression.

Notably, of the 36 proteins identified in the present study, the mRNA and protein expression of Jafrac1, p27k, arginine kinase, and paramyosin, which are the four known allergens, were significantly downregulated. Studies have shown that the four proteins can induce an IgE-mediated allergic response in patients (García-Orozco, Aispuro-Hernández, Yepiz-Plascencia, Calderón-de-la-Barca, & Sotelo-Mundo, 2007; Jeong et al., 2016; H. Wang et al., 2016; Zhao, Li, Kuang, Luo, & Li, 2015). Moreover, p27k and arginine kinase are thermostable, and thus, their

TABLE 2 Silkworm pupal proteins identified by mass spectrometry after 2-DE analysis

Spot	Protein name	Accession No.	Protein MW	Protein PI	Peptide count[a]	Protein score[b]	Ratio[c]
1	27-kDa glycoprotein	sp|Q8T113	25570.6	5.12	21	718	0.365
2	Thiol peroxiredoxin	tr|Q6T3A7	22073.2	6.09	17	525	0.675
3	Uncharacterized protein	tr|H9J343	21994.5	4.45	10	100	0.812
4	32-kDa apolipoprotein	tr|B9VTR5	32299.2	4.79	14	445	0.843
5	Paramyosin	tr|B3VTP0	10275.1	5.43	22	366	0.722
6	40S ribosomal protein	tr|H9JCR4	33487.9	4.86	11	288	0.794
7	Uncharacterized protein	tr|H9J4I1	27485.2	5.53	15	341	0.832
8	Antitrypsin isoform 3	tr|C7ASM6	43138.1	4.95	16	465	0.522
9	ATP synthase subunit beta	tr|Q1HPT0	54858.8	5.32	26	1010	1.649
10	Actin-4	tr|S5MNU1	42162	5.3	22	846	1.248
11	Antichymotrypsin-2	sp|P80034	41432.3	5.26	18	485	0.892
12	Uncharacterized protein	tr|H9JTV6	51536.6	5.85	15	772	0.730
13	Actin-4	tr|S5M683	42166.9	5.22	22	926	0.795
14	Putative peptidase	tr|D2KMR2	78076.4	6.37	18	289	0.723
15	Elongation factor 1-α	tr|H9J271	50698.6	9.21	18	620	1.987
16	Imaginal disk growth factor	tr|A7BEX9	48361.9	7.64	5	61	0.877
17	Uncharacterized protein	tr|H9J4I1	27485.2	5.53	8	486	0.654
18	Arginine kinase	tr|Q2F5T5	39991.3	5.87	25	942	0.612
19	Actin-4	tr|S5MI94	42221.9	5.3	23	893	1.248
20	Uncharacterized protein	tr|H9IXK0	41892.7	5.14	20	576	0.765
21	Uncharacterized protein	tr|H9JP90	35370.7	5.95	13	722	1.024
22	Tropomyosin-1	sp|Q1HPU0	32602.6	4.78	21	343	0.865
23	Vitellogenin	tr|Q33CE5	203420	6.99	33	782	0.805
24	Uncharacterized protein	tr|H9J8H0	50638.6	6.26	24	211	0.758
25	Uncharacterized protein	tr|H9IVS0	38982.4	5.79	15	308	0.747
26	Low-molecular 30-kDa lipoprotein PBMHPC-23	sp|P09338	30609.2	8.46	27	915	0.554
27	Phosphoribosyl aminoimidazole carboxylase	tr|Q1HQ66	47656.7	8.08	14	232	0.759
28	Fructose-bisphosphate aldolase	tr|Q1HPN7	39970.8	8.38	21	447	0.844
30	Nucleoside diphosphate kinase	tr|Q1HPI5	17358.9	6.75	11	421	0.898
31	30-kDa protein	tr|C7A8A2	29814.9	5.92	15	727	0.829
32	Peptidyl-prolyl isomerase	tr|Q5CCL3	18118.1	7.74	10	436	1.336
33	Uncharacterized protein	tr|H9JWX5	195519.9	8.95	32	414	0.638
34	Kinesin-like protein	tr|H9JBB4	116066.9	5.84	24	67	0.840
35	Uncharacterized protein	tr|H9JWX6	179221.5	8.93	30	297	0.791
36	Uncharacterized protein	tr|H9JPS0	113769.3	4.84	18	1040	0.662
37	Serine proteinase-like protein	tr|Q1HPQ5	44115.9	5.58	23	796	0.793
29	Unidentified						

Note. 2-DE: two-dimensional gel electrophoresis; MW: molecular weight; PI: isoelectric point.

[a]Mass of all peptides produced by mass spectrometry, which matches the number of peptides of the above proteins. This value reflects the coverage of the peptide.

[b]In the MASCOT algorithm, this indicates the score value and statistical reliability of getting this score.

[c]Infected/uninfected.

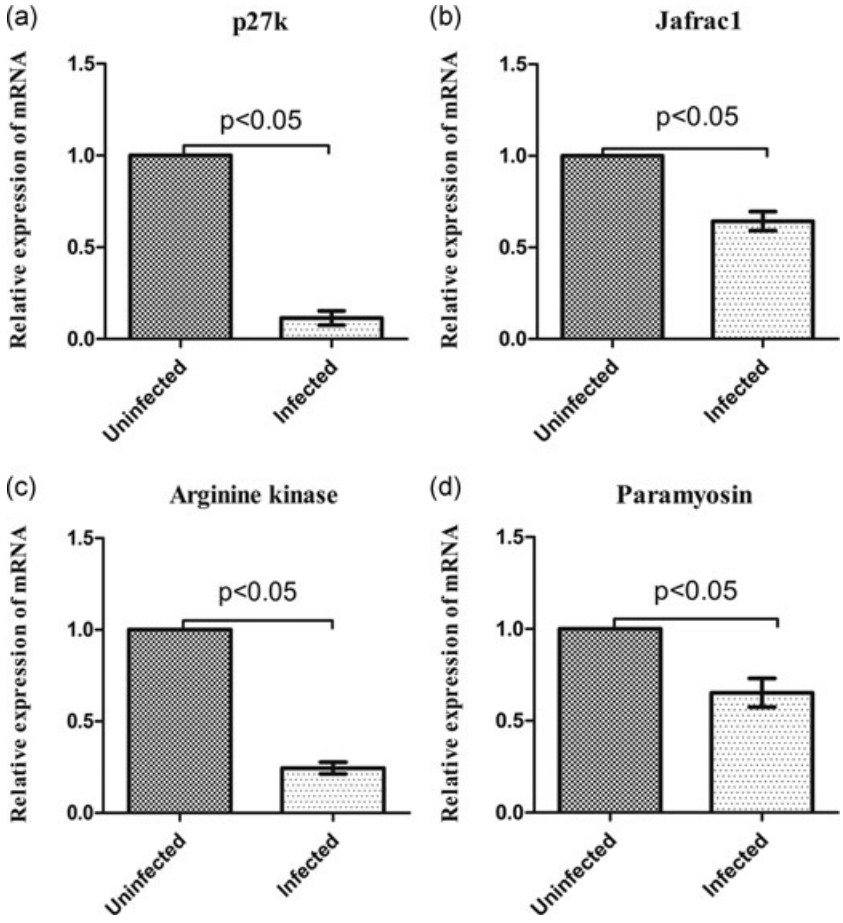


FIGURE 2 RT-qPCR analysis of the mRNA expression of the four allergens in infected and uninfected silkworm pupae ($n = 3$). (a) Relative expression of p27k in uninfected and BmNPV-infected pupae. (b) Relative expression of Jafrac1 in uninfected and BmNPV-infected pupae. (c) Relative expression of arginine kinase in uninfected and BmNPV-infected pupae. (d) Relative expression of paramyosin in uninfected and BmNPV-infected pupae. The relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method. Statistical analysis (Student's *t* test) was conducted using the GraphPad Prism software. BmNPV: *Bombyx mori* baculovirus; mRNA: messenger RNA; RT-qPCR: quantitative reverse-transcription polymerase chain reaction

allergenicity cannot be destroyed by heating (García-Orozco et al., 2007; Jeong et al., 2016). Madero et al. (2007) showed that the 27-kDa hemolymph protein of *Galleria mellonella*, an airborne allergen, shares 54.9% amino acid similarity with p27k of the silkworm, further highlighting its allergenicity. On the basis of these studies, the four proteins likely play a significant role in the observed allergic response to silkworm pupae when used as a drug carrier or protein expression bioreactor.

In a previous analysis by Bey et al. (1993), the p27k protein was shown to have multiple phosphorylation sites, an nucleotide triphosphate binding fold, and an RNA-binding consensus sequence. However, beyond this characterization and after localization studies in other species, very little is known about this protein. Though p27k appears to be a stable allergen (Jeong et al., 2016; Madero et al., 2007), additional studies are necessary to understand the mechanism underlying these effects.

Arginine kinase, a group of enzymes involved in energy catabolism, is found only in invertebrates and is the key to energy metabolism in invertebrates. It catalyzes the reversible transfer of high-energy phosphate from arginine phosphate to adenosine diphosphate to form adenosine triphosphate (ATP), thereby regenerating ATP during cell

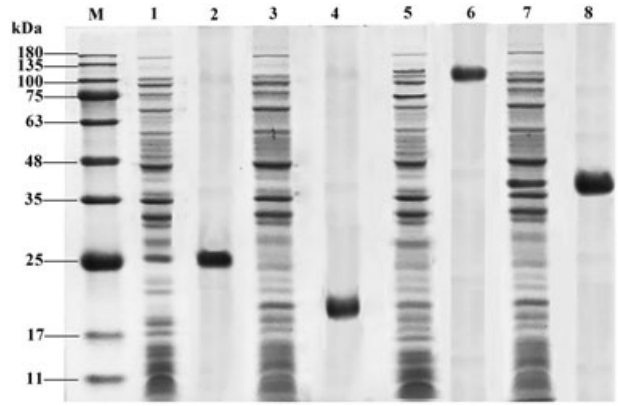


FIGURE 3 SDS-PAGE analysis of the recombinant proteins of the four allergens. Lane M: protein marker (11–180 kDa); Lane 1: unpurified supernatant containing recombinant p27k; Lane 2: purified recombinant p27k; Lane 3: unpurified supernatant containing recombinant Jafrac1; Lane 4: purified recombinant Jafrac1; Lane 5: unpurified supernatant containing recombinant paramyosin; Lane 6: purified recombinant paramyosin; Lane 7: unpurified supernatant containing recombinant arginine kinase; Lane 8: purified recombinant arginine kinase. Each lane of the 12% polyacrylamide gel contained 30 μg of each protein sample and was stained with Coomassie Brilliant Blue R250. SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

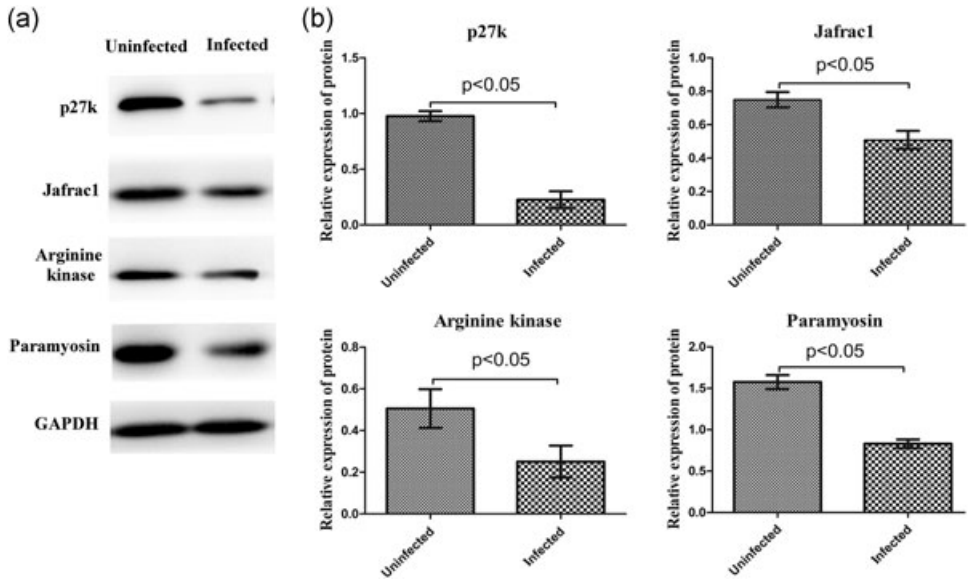


FIGURE 4 The expression of the four allergen proteins is downregulated in silkworm pupae after BmNPV infection. (a) Representative images of the western blot membrane showing the expression of the four allergen proteins in uninfected and infected silkworm pupae. (b) Quantitative analysis of the expression of the four allergen proteins in uninfected and infected silkworms after normalization to GAPDH, ($n = 3$), Student's t test. BmNPV: *Bombyx mori* baculovirus; GAPDH: glyceraldehyde 3-phosphate dehydrogenase

activity bursts (Yao, Wu, Xiang, & Dong, 2005). Except for silkworm pupae, it is also a pan-allergen in other invertebrates, such as moth (Binder et al., 2001) and lobster (Reddy, Roustan, & Benyamin, 1991). Therefore, in the silkworm pupa infected by BmNPV, although the downregulation of arginine kinase inhibited the formation of host ATP, the sensitization of silkworm pupa was reduced.

Paramyosin, a myosin filament-related protein found in the striated muscle of invertebrates, plays an important role in the process of myosin filament assembly (Elfvin, Levine, & Dewey, 1976) and mainly acts as a major muscle component in invertebrates. In addition, paramyosin was reported to possibly regulate the host immune responses and further induce allergenic reactions in humans (Pérez-Pérez et al., 2000). Therefore, the downregulation of paramyosin may be related to the host's antiviral immune response, and at the same time, as an important allergen protein, its downregulation also reduces the sensitization of silkworm pupa.

Jafrac1 is distributed in the extracellular region and participates in a variety of molecular functions, such as biometabolism, antioxidative stress, and biocatalysis. As a member of the peroxidase family, Jafrac1 can catalytically reduce hydrogen peroxide and alkyl hydroperoxides (Chae, Chung, & Rhee, 1994; Chae, Kim, Kim, & Rhee, 1993). It has also been shown to eliminate hydrogen peroxide production and inhibit viral antigen formation in host cells (El Messaoudi et al., 2002; Kang, Baines, & Rhee, 1998), thus preventing oxidative stress caused by viral infection (Lee et al., 2005). Moreover, Jafrac1 can also use thioredoxin as a direct hydrogen donor to reduce reactive oxygen species (Lim et al., 1993). Therefore, downregulation of Jafrac1 may result in increased oxidative stress in the host. In addition to its antioxidant effects, Jafrac1 also exhibits chaperone activity and serves as a redox sensor for regulating gene expression (Karplus & Hall, 2007; Veal, Day, & Morgan, 2007). Overexpression of Jafrac1 in *Drosophila* was previously shown to inhibit catalase sensitivity during immune regulation and prevent cell death and apoptosis from direct oxidative damage (Ha et al., 2005; Radyuk & Sohal, 2003). In addition, cell apoptosis and autophagy were shown to be an effective defensive response to baculovirus infection (Clem, 2005; Vaidyanathan & Scott, 2006; L. Wang et al., 2018). Therefore, downregulation of Jafrac1 may be closely related to the apoptosis and autophagy induced by the BmNPV vector. Taken together, BmNPV-mediated downregulation of Jafrac1 likely promotes the host's reaction to oxidative stress, accelerates virus infection, and shortens the host's life cycle. These characteristics, while detrimental to the silkworm, provide a theoretical basis for increasing the safety of silkworm pupal protein as a drug carrier material in addition to facilitating the replication of baculovirus vectors and expression of foreign proteins.

5 | CONCLUSION

In this study, we showed that the empty BmNPV vector inhibits the expression of four important allergens in silkworm pupae. These changes may reduce the allergen-related side-effects observed when using silkworm pupa-based drug delivery and protein expression. The known functions of these allergens in other processes, including accelerating viral infection and shortening the life cycle of the host, also highlight the practical benefits of using the BmNPV vector to express exogenous proteins or prepare the pupa for use as a drug delivery tool. These data provide a strong basis for further analysis of the mechanisms underlying hypoallergenicity in the silkworm bioreactor expression system as well as further development of efficient nonallergenic systems. This is the first study to demonstrate the effects of the empty BmNPV vector expression on allergen gene and protein expression in the silkworm, and along with future work, it will likely have a significant effect on how the silkworm expression system is used for protein expression and drug delivery in clinical settings.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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2.2. Protein preparation

The infected and uninfected silkworm pupae were crushed into a powder using a mortar with liquid nitrogen. A total of 150 mg of powder from each pupa was added to 1.5 mL of 10% TCA-acetone with protease inhibitors. The mixtures were then vortexed four times for 1 min each time every 15 min at –20°C and stored overnight. After centrifugation, the supernatant was discarded, and 1.5 mL of acetone was added, followed by centrifugation at 14,000 ×*g* for 1 h at 4°C. This process was repeated three times. Next, protein lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM, 0.2% DTT, in 1.5 mL of amphoteric electrolytes) was added, followed by static cracking at –20°C for 2 h. After centrifuging the samples at 14,000 ×*g* for 40 min at 4°C, a TCA-acetone protein purification kit (American CE) was used to remove salts, and the protein concentration was determined using the Bradford method.

2.3. Two-dimensional gel electrophoresis

Isoelectric focusing for two-dimensional gel electrophoresis (2-DE) was performed using an EttanIPGPhor3 (GE Healthcare, Little Chalfont, UK) with 13-cm gel strips, pH 3–10, and 150 μg of total protein from each sample. The gel strips were transferred to a prepared SDS-PAGE gel surface. The 12.5% gels were sealed with low-melting point agarose. Electrophoresis was performed using an SE600Ruby vertical electrophoresis tank (GE Healthcare) at 20 mA/min for approximately 4.5 h. After electrophoresis, the gel was removed; photofixed with 40% ethanol and 10% acetic acid for 1 h; washed twice with ultra-pure water; and stained with Coomassie Brilliant Blue dye for 6 h. After rinsing the stained gels with decolorizing liquid (10% acetic acid, 45% methanol), the gels were scanned until the background color disappeared. Spot detection, matching, and quantitative intensity analysis were performed using PDQuest software (version 8.0, Bio-Rad, Hercules, CA, USA).

2.4. Mass spectrometry

To identify the differentially expressed proteins in 2-DE analysis, we extracted colloidal particles from the gels and inserted them into microcentrifuge tubes. Next,

200–400 μL of 100 mM NH_4HCO_3/30% ACN was added to decolorize the gels until they were transparent. After removing the supernatant, 100 mM NH_4HCO_3 was added and incubated for 15 min at room temperature. The supernatant was then discarded, and the samples were freeze-dried. A total of 5 μL of 2.5–10 ng/μL sequencing Trypsin solution (Promega, Madison, WI, USA) was added and the samples were incubated at 37°C overnight. The enzyme solution was removed and transferred to a new microcentrifuge tube, while 100 μL of 60% ACN/0.1% TFA was added to the original tube. After ultrasonication for 15 min, the enzymatic hydrolysis product was freeze-dried. These freeze-dried samples were dissolved in 2 μL of 20% acetonitrile (Merck, Billerica, MA, USA). Next, 1 μL of the dissolved sample was added to the sample target and dried naturally at 20°C before adding 0.5 μL of saturation CHCA matrix solution (Sigma, St. Louis, MO, USA; solvent was 50% ACN, 0.1% TFA). After drying naturally, nitrogen gas was passed over the sample and the sample was placed in the target slot of a 4800 Plus MALDI TOF/TOF Analyzer (AB SCIEX, Framingham, MA, USA). For this analysis, we used positive ion mode, automatic data acquisition mode, and an MS scanning range of 800–4000 Da. Furthermore, a signal-to-noise ratio greater than 50 was used for parent ion tandem mass spectrometry (MS/MS) analysis, and 8 parent ions were selected for each sample point. The MS/MS was superimposed 2500 times, while the collision energy was set to 2 kV and CID was turned off. Mascot 2.2 software was used to analyze the results and identify the proteins.