

*Full Length Research Paper*

# Antiviral and antitumor activities of the protein fractions from the larvae of the housefly, *Musca domestica*

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In this study, we present the extraction and purification of protein fractions from the larvae of the housefly, *Musca domestica*. The bioactivities of the protein fractions were indicated by pseudorabies virus (PRV) and human lung cancer cell line A 549. The crude protein fractions had no toxicity to chick embryo fibroblast-like (CEF) cells but effective to PRV at the concentration of 100 µg/ml (dilute ratio of 1:100). Furthermore, they exhibited better antiviral effect when added to CEF cells 1 h after PRV infection than 1 h prior to PRV infection and could directly deactivate and hinder the adsorption or penetration of PRV into CEF cells. In addition, two major peaks (A1 and A2) were found by DEAE-52 from the crude protein fractions, but only the purified protein fraction A2 showed antiviral and antitumor activities. The results suggest that the protein fractions extracted and purified from the larvae of the housefly have antiviral and antitumor activities and can be further studied.

**Key words:** *Musca domestica*, antiviral, antitumor, protein.

## INTRODUCTION

The housefly, *Musca domestica* L. (Diptera: Muscidae) is a major domestic, medical, and veterinary pest that causes irritation, spoils food, and acts as a vector of many medical and veterinary pathogenic organisms (Förster et al., 2009). It lives in a habitat filled with opportunistic microorganisms from larva to adult, but is seldom being infected. This has risen up research interests for years. The larva, also called the maggot, has been used clinically to cure malnutritional stagnation, decubital necrosis, osteomyelitis, ecthyma and lip boil since the Ming/Qing Danysty (1368 Anno Domini) up to now in China, and also been used to treat coma and gastric cancer when combined with other drugs (Jiang, 1999; Hou et al., 2007a, 2007b). Antibacterial and immunoactive substances in *M. domestica*, such as prophenoloxidase, antibacterial protein/peptide, lysozyme and some other secretions have been studied (Lemos

and Terra, 1991; Lemos et al., 1993; Ito et al., 1995; Meylaers et al., 2004; Liang et al., 2006; Wang et al., 2006; Jin et al., 2006; Cançado et al., 2007; Xu et al., 2007; Hou et al., 2007a; Ai et al., 2008, 2012; Ren et al., 2009; Cao et al., 2011).

However, antiviral and antitumor proteins or peptides from *M. domestica* have been poorly reported except several reports recently. It is reported that antiviral components were existed in larvae homogenates of *M. domestica* and the authors speculated the antiviral active components may be proteins or peptides (Chen and Li, 2003; Li and Chen, 2006). Similarly, our lab found that antiviral substances were in the body of housefly larvae by utilizing the chicken embryos and HbsAg kit (Wang et al., 2006). So the objective of the present study was to extract the antiviral and antitumor active proteins from larvae of *M. domestica*.

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## MATERIALS AND METHODS

### Organisms

The housefly, *M. domestica* was cultured in our lab and the third

instar larvae were prepared for the experiments. Pseudorabies Virus (PRV) was obtained from the China Institute of Veterinary Drugs Control (Beijing, China). Human lung cancer cell line A 549 was bought from the China Center for Type Culture Collection of Wuhan University (Wuhan, China).

### The extraction of the crude protein fractions

To obtain the crude protein fractions, the third instar larvae were collected, washed with distilled water, frozen and lyophilized. Then, the lyophilized larvae were extracted with petroleum ether (boiling point, 30 - 60 °C) in a Soxhlet apparatus for 12 h. Later the defatted larvae were pulverized at low temperature. The defatted powder of the larvae was obtained and treated in cold buffer (0.18 mol/L NaCl citrate - Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0, 4°C) with a ratio of 1:3 (w/v) for 15 min. After centrifugation at 1, 800 g for 10 min, the supernatant was transferred to a new container and acidified to pH of 5.8 with HCl. Then, the extracts were subjected to graded precipitation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (below 65% saturation) to yield the insoluble fractions. In all cases, the precipitate was recovered by centrifugation and dissolved in ultrapure water prior to dialysis. All the dialyzed extracts were lyophilized and then the crude protein fractions were stored at -80°C.

### Biological activity of the crude protein fractions

#### The cytotoxicity of the crude protein fractions

The cytotoxic effect of the crude protein fractions was assessed by using chick embryo fibroblast-like (CEF) cells according to Greco et al. (2009) with slight modification. Briefly, 100 µl of CEF cell cultures were exposed to 100 µl of the crude protein fractions (dilution rates were 1:1, 1:10 or 1:100) on 96-well plates and then incubated at 37°C for different time. Daily, the cytopathic effect (CPE) was observed.

#### Determination of the virus infectious dose

In order to determine the virus infectious dose, CEF cell cultures were seeded at a concentration of 2×10<sup>5</sup> cells/ml on 96-well plates and each well was 100 µl. These cells were infected with 100 µl of PRV suspension of different concentration (10<sup>-1</sup> to 10<sup>-8</sup>) and then the microwell plates were incubated at 37°C, as described by Greco et al. (2009). The virus titer was determined by monitoring the CPE in an endpoint dilution assay. It was expressed as TCID<sub>50</sub> (the highest dilution of virus able to induce CPE in 50% of cells) and calculated by the method of Reed and Muench (1938).

#### The inactivating effect of the crude protein fractions on PRV

The inactivating effect of the crude protein fractions on PRV was performed according to Greco et al. (2009) with slight modification. CEF cell cultures were seeded at a concentration of 2×10<sup>5</sup> cells/ml on 96-well plates and each well was 100 µl. PRV suspension was mixed with 100 µg/ml of the crude protein fractions for 2 h and then 100 µl of the mixture were added to the microwell. Controls were only treated with PRV suspension. The microwell plates were then incubated at 37°C. Daily, the CPE was determined to find the inactivating effect of the crude protein fractions on PRV.

#### The effect of the crude protein fractions on the adsorption step

The supernatant of infected cultures was assayed for the remaining

virus to detect the effect of the crude protein fractions on the adsorption step as described by Greco et al. (2009). CEF cell cultures were supplemented with the crude protein fractions (100 µg/ml) 1 h or 2 h before virus infection and then infected with PRV suspension. Non-supplemented cell cultures were carried out under the same conditions as control. 2 h after infection, samples of supernatant were harvested and the virus titers were carried out on 96-well plates.

### The purification of the crude protein fractions

To purify the crude protein fractions, they were obtained as above (60 mg) and dissolved in 0.05 M Tris-HCl buffer (pH 8.0) and filtered with 0.45 µm membrane. The sample was loaded onto DEAE-cellulose column (2×20 cm) pre-equilibrated with 0.05 M Tris-HCl buffer (pH 8.0). The sample was eluted successively with two rinses of 0.05 M Tris-HCl buffer (pH 8.0) and 0.5 mol/L NaCl. The flow rate was set at 0.3 ml/min. Each 3-ml fraction was collected and monitored at 280 nm. Unless stated otherwise, all steps of purification were carried out at 4°C. The eluted retention fractions were vacuum concentration and stored at -80°C.

### Biological activity of the purified protein fractions

#### The cytotoxicity of the purified protein fractions

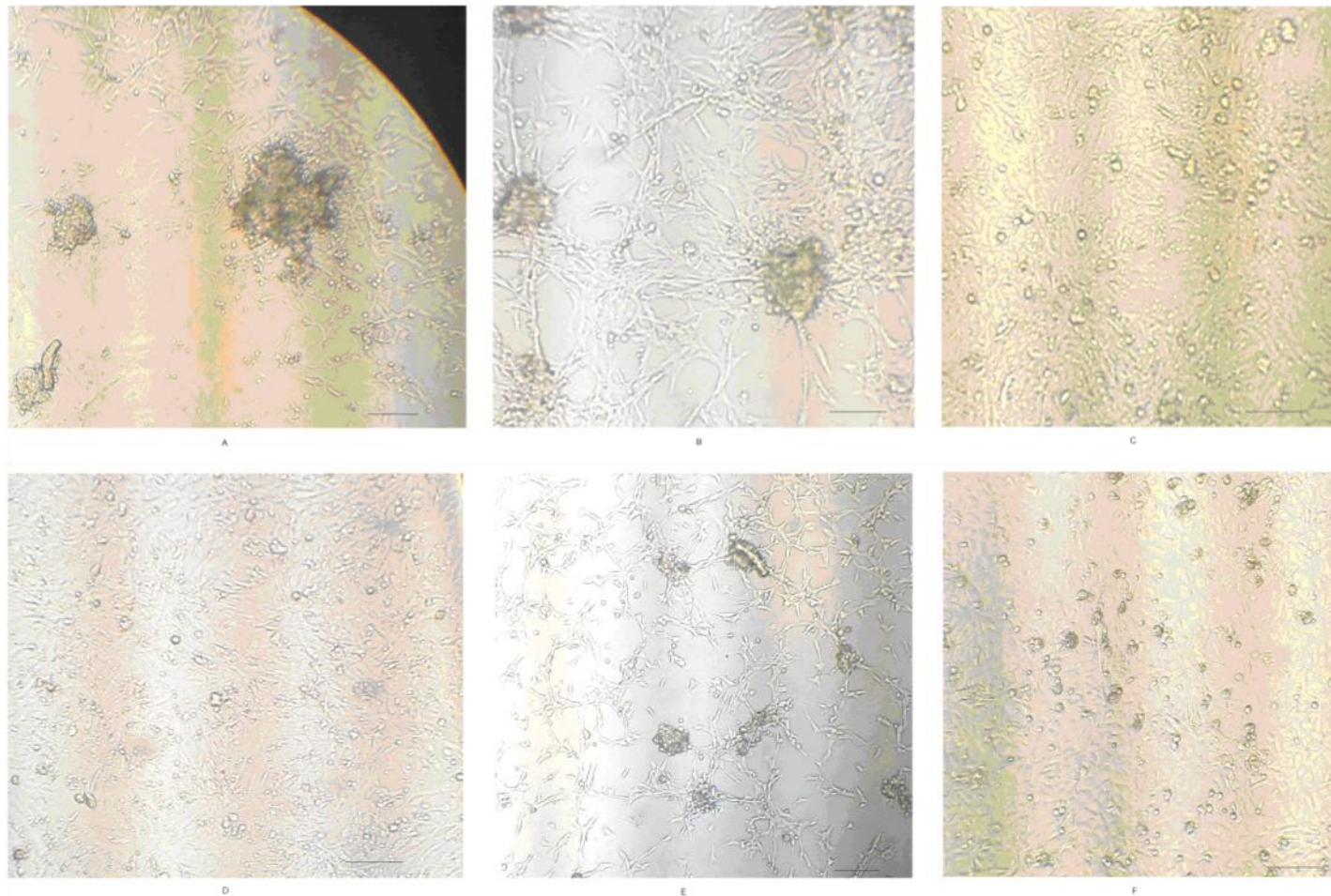
The cytotoxic effect of the purified protein fractions was assessed by using CEF cells according to Greco et al. (2009) with slightly modified. Briefly, 100 µl of CEF cell cultures were exposed to 100 µl of the purified protein fractions of a series of concentration (5000, 1000, 200, 40, 8, 1.6, 0.32 or 0.064 µg/ml) on 96-well plates and then incubated at 37°C for different time. Daily, the CPE was observed.

#### The antiviral activity of the purified protein fractions

The antiviral activity of the purified protein fractions was performed as described above. CEF cell cultures were seeded at a concentration of 2×10<sup>5</sup> cells/ml on 96-well plates and each well was 100 µl. PRV suspension was mixed with the purified protein fractions for 2 h and then 100 µl of the mixture were added to the microwell. Controls were only treated with the PRV suspension. Then, the microwell plates were incubated at 37°C. Daily, the CPE was determined to confirm the inactivating effect of the purified protein fractions on PRV.

#### The antitumor activity effect of the purified protein fractions

The *in vitro* inhibitory effect of the purified protein fractions on human lung cancer cell line A 549 was performed according to Hou et al. (2007b) with some modifications. Cells in dimethyl sulfoxide (DMSO) with 10% fetal bovine serum was plated on 96-well plates (5×10<sup>4</sup> cells/well) and allowed to adhere at 37°C, and 5% CO<sub>2</sub>. The monolayer of cells in the plate was exposed to various dilutions of the purified protein fractions (6, 3, 1.5, 0.75, 0.375, 0.1875, 0.093 or 0.0468 mg/ml). Then, the cells were incubated at 37°C, and 5% CO<sub>2</sub> for 48 h. Controls were maintained throughout the experiments (untreated wells as the cell control and diluent treated wells as the substrate control). After incubation, 0.5% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added into each well and then the wells were incubated at 37°C, and 5% CO<sub>2</sub> for 4 h. Later, the supernatant after centrifuging at 1000 g for 5 min was discarded (DMSO) (100 µl/well) was added to dissolve the formazan crystals formed. After solubilizing, the



**Figure 1.** The cytopathic effect of the crude protein fractions from the larvae of *Musca domestica* on chick embryo fibroblast-like cells (bar = 100  $\mu\text{m}$ ). A, The cells were exposed to 1-fold dilution of the crude protein fractions for 1 day; B, the cells were exposed to 10-fold dilution of the crude protein fractions for 1 day; C, the cells were exposed to 100-fold dilution of the crude protein fractions for 1 day; D, control (use the DMEM medium with the crude protein fractions and serum free as control); E, the cells were exposed to 1-fold dilution of the crude protein fractions for 2 days; F, the cells were exposed to 10-fold dilution of the crude protein fractions for 2 days.

optical density (OD) of each well was detected by the microplate reader at 490 nm. The inhibitory rate (IR) was calculated as follows:

$$\text{IR (\%)} = (\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}) / \text{OD}_{\text{control}} \times 100$$

## RESULTS

### The cytotoxicity of the crude protein fractions

As described previously, CEF cells were exposed to different concentration of the crude protein fractions and the cytotoxic effect was detected. Figure 1 shows the CPE of the crude protein fractions on CEF cells. The cells treated by the crude protein fractions with dilute ratio of 1:1 and 1:10 for 1 day became contracted and the netting phenomenon appeared (Figure 1A, 1B). However, the crude protein fractions with the dilute ratio of 1:100 were not toxic to the CEF cells (Figure 1C). Two days later, the

CEF cells treated by the crude protein fractions with the dilute ratio of 1:1 partly recovered (Figure 1E), while those cells treated by the crude protein fractions with the dilute ratio of 1:10 could completely regrow (Figure 1F). Based on these, we indicate that the optimal concentration for crude protein fractions was 100  $\mu\text{g/ml}$ , that is, dilute ratio of 1:100.

### The inactivating effect of the crude protein fractions on PRV

In order to detect the direct inactivating effect of the crude protein fractions on the virus, PRV was exposed to 100  $\mu\text{g/ml}$  of the crude protein fractions for 2 h before infection. As indicated in Table 1, the virus titer of the crude protein fractions added 1 h before virus infection was  $3.16 \times 10^4$  TCID<sub>50</sub>/ml, while it was  $1.0 \times 10^4$  TCID<sub>50</sub>/ml 1 h after virus infection; both were significantly different

**Table 1.** The inactivating effect of the crude protein fractions from the larvae of *Musca domestica* on pseudorabies virus.

Treatment	PRV (control)	The crude protein fractions added	
		1 h before PRV infection	1 h after PRV infection
TCID <sub>50</sub> /ml	5.62×10 <sup>4</sup>	3.16×10 <sup>4</sup> **	1.0×10 <sup>4</sup> **

TCID<sub>50</sub>, tissue culture infectious dose 50%; PRV, pseudorabies virus. The values represent the average of three replications. Asterisks denote significant differences; \*\*P<0.01.

**Table 2.** Effect of the crude protein fractions from the larvae of *Musca domestica* on pseudorabies virus's adsorption step.

The supernatant of PRV infected cultures	Supplemented with the crude protein fractions		
	1 h before PRV infection	2 h before PRV infection	no supplement (control)
TCID <sub>50</sub> /ml	5.62×10 <sup>4</sup> **	2.51×10 <sup>4</sup> **	3.98×10 <sup>3</sup>

TCID<sub>50</sub>, tissue culture infectious dose 50%; PRV, pseudorabies virus. The values represent the average of three replications. Asterisks denote significant differences; \*\*P<0.01.

than that in the control (5.62×10<sup>4</sup> TCID<sub>50</sub>/ml). So, the crude protein fractions seemed to have some direct deactivating effect on PRV.

### Effect of the crude protein fractions on inhibiting PRV absorption

The virus remaining in the supernatant of PRV infected cultures with or without the crude protein fractions was detected (Table 2). The virus titer of the control (the supernatant without the crude protein fractions) was 3.98×10<sup>3</sup> TCID<sub>50</sub>/ml. Compared with the control, there was a significant difference when the supernatant was supplemented with the crude protein fractions 1 h or 2 h before virus infection (5.62×10<sup>4</sup> and 2.51×10<sup>4</sup> TCID<sub>50</sub>/ml, respectively). In addition, a significant difference was recorded in the virus titer between the two treated groups. We could conclude that the crude protein fractions can hinder the adsorption or penetration of the virus into cells and the supplemented time for the crude protein fractions can influence the hindering effect.

### Purification of the crude protein fractions

The crude protein fractions were analyzed by DEAE-52 to isolate and purify the antiviral protein. Two major peaks were obtained (Figure 2, A1 in tube 16 and A2 in tube 66). The experiment conditions were as follows: the column was pre-equilibrated with 0.05 M Tris-HCl buffer (pH 8.0). The sample was eluted by the above buffer and then by 0.05 M Tris-HCl buffer (pH 8.0) with 0.5 mol/L NaCl at a flow rate of 0.3 ml/min. Fractions of 3.0 ml each time were collected and analyzed for protein at 280 nm. The eluted major peak fractions were vacuum concen-

tration and stored at -80°C.

### The cytotoxicity of the purified protein fractions

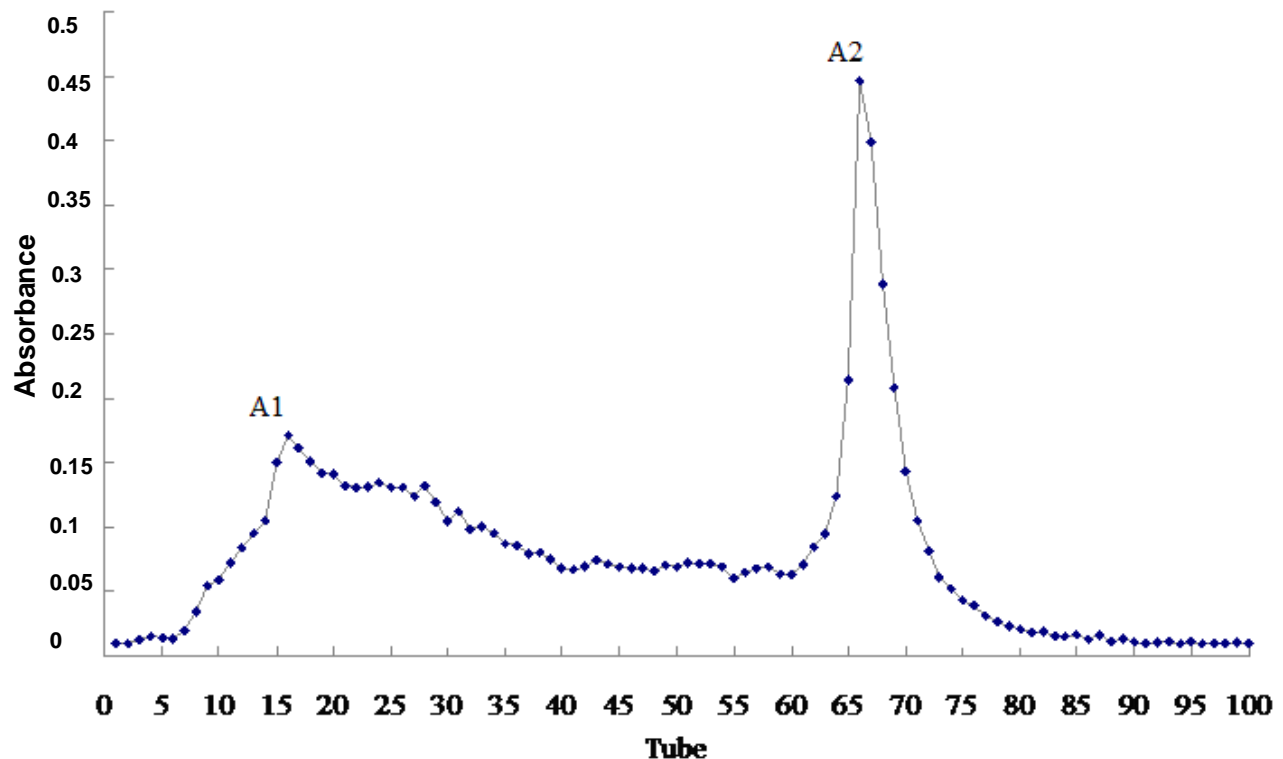
CEF cells were exposed to the purified protein fractions and the cytotoxic effect was determined as described previously. Within 24 h, the purified protein fraction A1 was toxic at 5 mg/ml and A2 was toxic at 5 mg/ml and 1 mg/ml, but both were not toxic at 0.2 mg/ml. Furthermore, the CEF cells treated by A2 (5 mg/ml and 1 mg/ml) were partly recovered after 48 h. Based on these; the concentration of 0.2 mg/ml was selected to use in the following experiments.

### Antiviral activity of the purified protein fractions

PRV was exposed to 0.2 mg/ml of the purified protein fractions for 2 h before infection. As indicated in Table 3, compared with the control (5.62×10<sup>4</sup> TCID<sub>50</sub>/ml), there was a significant decline (3.75×10<sup>4</sup> TCID<sub>50</sub>/ml) for the virus titer in the treatment of the purified protein fraction A2. However, it had almost no effect (5.62×10<sup>4</sup> TCID<sub>50</sub>/ml) for the purified protein fraction A1 on the virus titer.

### Antitumor activity of the purified protein fractions

The *in vitro* inhibitory effect of the purified protein fractions on A 549 tumor cells was investigated by the standard MTT colorimetric method. As shown in Table 4, the purified protein fraction A1 exhibited little inhibitory effect on the growth of A 549 tumor cells. On the contrary, inhibitory rate of the purified protein fraction A2 on A 549 tumor cells reached 66.5 and 51.1%, at 6 mg/ml and 3 mg/ml, respectively (Table 5). A dose-dependant manner



**Figure 2.** Elution profile of the crude protein fractions from the larvae of *Musca domestica* assayed by DEAE-52 column. The crude protein fractions were analyzed by DEAE-52 with 0.05 M Tris-HCl buffer (pH 8.0) and 0.5 mol/L NaCl at a flow rate of 0.3 ml/min.

**Table 3** Antiviral activity of the purified protein fractions from the larvae of *Musca domestica* on pseudorabies virus.

Treatments	PRV (control)	PRV with the purified protein fraction	
		A1	A2
TCID <sub>50</sub> /ml	5.62×10 <sup>4</sup>	5.62×10 <sup>4</sup>	3.75×10 <sup>4</sup> **

TCID<sub>50</sub>, tissue culture infectious dose 50%; PRV, pseudorabies virus. The values represent the average of three replications. Asterisks denote significant differences; \*\*P<0.01.

**Table 4.** Antitumor activity of the purified protein fraction A1 from the larvae of *Musca domestica* on A 549 tumor cell.

Parameter	Concentration of A1 (mg/ml)							Cell Control	Substrate Control	
	6	3	1.5	0.75	0.375	0.187	0.093			0.046
OD	0.612	0.667	0.654	0.645	0.641	0.642	0.659	0.668	0.695	0.080
IR (%)	12.1	4.1	6.0	7.2	7.9	7.7	5.3	4.0	/	/

The values represent the average of three replication. IR (%) = (OD<sub>control</sub> - OD<sub>sample</sub>) / OD<sub>control</sub> × 100.

was also observed in this antitumor activity assay.

## DISCUSSION

The extraction and purification of antibacterial and

immunoactive substances from *M. domestica* have been widely reported. For instance, a bacteria-digesting midgut-lysozyme from *M. domestica* was first purified by Lemos and Terra (1993) and 1-lysophosphatidylethanolamine (C16:1) as an antimicrobial compound was

**Table 5** Antitumor activity of the purified protein fraction A2 from the larvae of *Musca domestica* on A 549 tumor cell.

Parameter	Concentration of A2 (mg/ml)								Cell Control	Substrate Control
	6	3	1.5	0.75	0.375	0.187	0.093	0.046		
OD	0.235	0.342	0.407	0.427	0.465	0.521	0.583	0.651	0.699	0.078
IR (%)	66.5	51.1	41.9	39.0	33.5	25.5	16.7	7.0	/	/

The values represent the average of three replications. IR (%) =  $(OD_{\text{control}} - OD_{\text{sample}}) / OD_{\text{control}} \times 100$ .

purified by Meylaers et al. (2004). The chitosan, extracted from *M. domestica* was found to have effect on fungus and bacteria (Ai et al., 2008, 2012) while the extract from the larvae of the housefly exhibited antibacterial activity and *in vitro* anti-tumor activity (Hou et al., 2007a). In addition, Hf-1, a novel antibacterial peptide, was also purified by Hou et al. (2007b). Lectin, which can up-regulate NO and iNOS production via TLR4/NF- $\kappa$ B signaling pathway in macrophages was purified from *M. domestica* pupa (Cao et al., 2011). In this study, the protein fractions with antiviral and antitumor activities were extracted and purified from the larvae of *M. domestica*.

As shown by a series of biological assays, the crude protein fractions (i) had no toxicity to CEF cells but effective to PRV at the concentration of 100  $\mu$ g/ml (dilute ratio of 1:100), (ii) showed better effect when added to the CEF cells 1 h after PRV infection than 1 h prior to PRV infection, (iii) had some direct deactivating effect on PRV, and (iv) could avoid the adsorption or penetration of PRV into the CEF cells. Two major peaks (A1 and A2) were seen when the crude protein fractions were analyzed by DEAE-52 with 0.05 M Tris-HCl buffer (pH 8.0) and 0.5 mol/L NaCl at a flow rate of 0.3 ml/min (Figure 2). The purified protein fraction A1 exhibited little effect on virus PRV (Table 3) and tumor cells A549 (Table 4), while antiviral and antitumor activities were seen on the purified protein fraction A2 (Table 3, 5).

There may be a number of antiviral resistance mechanisms for proteins or peptides from insects. The alloferon from the fly *Calliphora vicina* can effectively inhibit influenza viruses A and B reproduction by triggering intracellular responses when added before virus infection (Chernysh et al., 2002), while the antiviral peptide in *Heliothis virescens* hemolymph may be directed to the inner leaflet of the plasma membrane thus blocking virus assembly and/or budding and exit of viruses from a host cell (Resh, 1999; Ourth, 2004). For *Lonomia obliqua* protein, it may act either on the steps of the cycle of replication of viruses that occur intracellularly, similarly to alloferon, or on the late stages of virus infection, similarly to the peptide from *H. virescens* (Greco et al., 2009). Furthermore, it seems to be unable either to inhibit the stages before reaching the target cell or to prevent the process of adsorption (Greco et al., 2009). Interestingly, another antiviral mechanism for proteins or peptides may be found in our experiments. The protein fractions isolated from *M. domestica* had

some direct deactivating effect on the tested virus and can act on the adsorption or penetration of the virus.

In conclusion, the protein fractions extracted and purified from the larvae of the housefly have antiviral and antitumor activities, which support their use in traditional Chinese medicines and can be further studied.

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