Anti Pseudorabies Virus Activity of Kumazasa Extract

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Sasa veitchii or "kumazasa" has been used for the preservation of food, or preventing bacterial activity. However, the antiviral activity of kumazasa is poorly understood. In the present study, the antiviral activity of kumazasa extract (KE) was assessed by the plaque reduction assay for the pseudorabies virus (PRV). KE reduced 99% of the plaque formation of PRV at concentrations of 1.2%, showing that KE inhibited PRV adsorption to cells and IE180 expression. The polysaccharide fraction of KE showed a concentration dependent inhibition of PRV plaque formation. We conclude that KE possesses potent anti PRV activity, and the candidate responsible for the antiviral property was the polysaccharide fraction.

Key words: Antiviral activity/Kumazasa/Medicinal plant/Pseudorabies virus/Polysaccharide.

INTRODUCTION

The pseudorabies virus (PRV) is a member of the alphaherpesvirus subfamily, a swine herpesvirus type 1, and is related to other animal pathogens such as bovine herpesvirus types 1 and 5, and equine herpesviruses types 1 and 4. In addition, the alphaherpesvirus subfamily includes human pathogens herpes simplex virus type 1 (HSV-1), HSV-2 and varicella-zoster virus (Roizman & Pellett, 1991). PRV infection is characterized by different symptoms and outcomes of infection in natural and nonnatural hosts. In natural hosts, young pigs are more severely affected by PRV infection, often resulting in fatal encephalitis, but older infected pigs might remain asymptomatic, or develop mild to severe respiratory disease symptoms associated with limited mortality (Pomeranz et al., 2005). A limited number of PRV vaccines, which are either attenuated or inactivated viruses, are available on the market. However, both of these types of vaccines have inherent drawbacks. Vaccination strategies alone usually suppress manifestation of the disease but do not stamp out viral infection from a population.

Many plant extracts have been described as potential antiviral agents (De Logu et al., 2000; Minami et al., 2003; Akanitapichat et al., 2006; Leung et al., 2006; Gu et al., 2007). Recent reports show interesting results of the antiviral activity of plant extracts in experimental and clinical medicine (Venkateswaran et al., 1987; Reichling et al., 2005). For some time Kumazasa has been used for the preservation of food, or preventing bacterial activity. Again there are a variety of pharmacologic actions also in the extract. Kumazasa has been reported to exhibit various biological functions such as antibacterial activity, anti-ulcer property, cell repair function and anti-tumor action (Yamamoto et al., 1971; Otani et. al., 1990;
Endo et al., 1994; Tsunoda et al., 1998; Ren et al., 2004).

The present study investigated the antiviral activity of KE against PRV. Moreover, the candidate for the portion responsible for its antiviral property was the polysaccharide fraction of KE.

MATERIALS AND METHODS

Kumazasa extract and preparation of the polysaccharide fraction

Kumazasa extract (KE) was extracted from kumasasa leaf with hot water. Lyophilization and measurement of the dry weight of KE contained 640 ± 4mg solid material/ml. The KE was precipitated by 40 and 80% ethanol sequentially at 4°C overnight, and the pellet was dissolved in distilled water and lyophilized as a powder herein called the kumazasa polysaccharide fraction (KPF).

Cells and virus

African green monkey kidney cells (Vero) were grown in Eagle’s minimum essential medium (MEM) supplemented with 5% fetal calf serum (FCS), 29.2 μg/ml glutamine, 2.95 μg/ml tryptose phosphate broth (TPB), 75 μg/ml NaHCO₃, 20 Unit/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% (v/v) CO₂ incubator at 37°C. The strain of PRV YS-81 was used in this study.

Cytotoxicity test

Cytotoxicity of KE and KPF were tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Vero cells in 96-well culture plates were exposed to MEM containing a serial dilution of KE. After treatment for 48 h, the cells were incubated with 50 μl of MTT (0.5mg/ml) at 37°C for 4 h. After the removal of supernatant, 50 μl of DMSO was added to solubilize the formazan crystals formed, and the optical densities at 550 nm were measured using a microplate reader. The 50% cytotoxic concentration (CC₅₀) was the concentration of the KE required to reduce the optical densities of mock-treated wells by 50%.

Plaque reduction assay

Confluent Vero cells in 6 well plates were infected with 100 PFU of PRV with serial dilutions of 1.2% KE for 1 h at 37°C. After the removal of the supernatant, the inoculum was replaced with MEM containing 0.8% agarose and serial dilutions of 1.2% KE. Infected cultures were incubated in a humidified 5% CO₂ incubator for 3 days. Then the infected cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Then the fixed cells were stained with 0.8% (w/v) crystal violet in 50% ethanol and then viral plaques were counted. As the control, the assay was carried out similarly without KE. The reduction in the number of plaques was calculated as the percentage of that of the virus control. The concentration reducing plaque formation by 50% relative to the control was estimated from graphic plots and defined as the 50% inhibitory concentration (IC₅₀).

Fluorescent antibody (FA) technique

Vero cells in 6-well culture plates with a cover glass were infected with PRV diluted in MEM to MOI=1 for 15h at 37°C in the presence or absence of 1.2% KE. After 15 h incubation the infected cells were fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. Then the infected cells were washed 1 time each with PBS, and cells were permeabilized with 0.05% Triton X-100 in PBS for 30 minutes at room temperature. For 30 minutes blocking was done at room temperature in 1% Skim milk with PBS. Then the infected cells were incubated with rabbit anti PRV antibodies diluted in 1% Skim milk with PBS for 60 min at 37°C, washed with PBS, and then incubated with FITC labeled goat anti rabbit antibodies diluted in 1% Skim milk with PBS for 60 min at 37°C. The samples were washed three-times with PBS and examined for fluorescence.

Direct virus neutralization test

Direct virus neutralizing activity was determined by incubating 1.2% KE with 100 PFU of PRV for 1 h at 37°C. Vero cells were infected with KE/PRV mixture and incubated for 1 h at 37°C. After the removal of supernatant, infected cells were washed with PBS in duplicate and overlaid with MEM 0.8% agarose without KE. After 72h cells were fixed and stained as described above.

Adsorption assay

Attachment assays were performed using published procedures with modifications (De Logu et al., 2000). Vero monolayers grown in 6-well plates were pre-chilled at 4°C for 15min and infected with PRV diluted in MEM to 100 PFU for 3h at 4°C in the presence or absence of 1.2% of KE. The inoculum was then removed and washed 2 times with PBS. Cells were overlaid with MEM-0.8% agarose. After 72 h the cells were fixed and stained as described above.

Penetration assay

Penetration assays were performed using published procedures with modifications (De Logu et al., 2000). Briefly, about 100 PFU of PRV was adsorbed
for 3 h at 4°C on Vero cells grown on 6-well plates. The temperature was then abruptly increased to 37°C to maximize the penetration of the virus. Penetration proceeded for 1 h in the absence or presence of Brix 1.2% KE. Monolayers were then treated with PBS, pH 3 for 1 min to neutralize any remaining attached virus and, after being washed two times with serum-free medium cells, were overlaid with MEM containing 0.8% agarose without KE. After 72 h cells were fixed and stained as described above.

**Western blot analysis of IE180 expression**

The expression of IE180 was examined using western blot analysis. After Vero cells were infected with PRV (MOI = 1) for 1h, cells were treated with 1.2% KE or left untreated. After 24, 48 and 72 h, PRV infected cells were boiled for 5min in the sample buffer before electrophoresis on 10% SDS polyacrylamide gel. Following electrophoresis, proteins were transferred to Immobilon Transfer Membrane (Millipore, MA, USA). The membrane was treated sequentially with 1% Skim milk with anti IE180 rabbit serum (Tomioka et al., 2008) and finally, HRP conjugated anti rabbit IgG (BIORAD). Internal controls were carried out simultaneously by detecting β-actin with anti β-actin antibodies. Antigen was detected using the TMB membrane peroxidase substrate system (KPL, MD, USA).

**Statistical methods**

The stage of inhibition of KE, the attachment and the penetration assays were analyzed using a t-test. A $P$ value less than 0.05 was considered significant.

**RESULTS**

**Cytotoxicity**

The C50 of KE and KPF against Vero cells was determined by the MTT assay. The C50 value of KE was calculated as 2.4%, and the maximum concentration which showed non-cytotoxicity on Vero cells by the MTT assay was 1.2% (data not shown). As a result, this concentration, 1.2%, was used in the antiviral assays. The C50 values of KPF was calculated as 10mg/ml.

**Antiviral activity of KE**

The antiviral activity of KE against PRV was first evaluated by a plaque reduction assay using Vero cells. The plaque reduction assay showed a dose-dependent antiviral activity of KE against PRV. 99% inhibition was observed at a concentration of 1.2%, and 36.4% inhibition was still observed at 0.15% (Fig.1). IC50 of KE was determined to be 0.24%.

Since it turned out that there was an antiviral activity, in order to investigate whether there was any difference also in the number of infected cells, the FA technique was performed. Reduction was seen again in the number of infected cells in the FA technique (Fig. 2). However, KE did not have virucidal effect on PRV (Fig. 3A)

**Attachment and Penetration assays**

To assess the stage of inhibition of KE, the attachment and a penetration assays were performed. Attachment assays indicated a 25% inhibition in comparison to the untreated control at a concentration of 1.2 % (Fig. 3B). On the other hand, no inhibition was observed when Vero cells were infected with PRV at 4°C to prevent penetration of viruses and the temperature was then increased to 37°C under treatment with KE (Fig. 3C).

**KE suppressed IE180 expression**

Results of the FA assay suggested that KE suppressed PRV gene expression. To determine the

![FIG. 1. Antiviral activity of KE against PRV as determined by the plaque reduction assay. Vero cells were infected with 100 PFU of PRV treated with serially diluted KE. The data represent the mean of three separate experiments.](image)

![FIG. 2. Effect of KE on PRV antigen expression (Immunofluorescence assay). PRV infected cells were treated with KE (A) or virus control (B).](image)
immediate early 180 (IE180) expression in the infected cells with or without KE treatment, (1.2%), expression levels of IE180 were analyzed by the western blot (Fig. 4). IE180 were expressed in KE untreated PRV infected Vero cells at all times tested. On the other hand, IE180 expression was suppressed on PRV infected cells treated with KE at 24h and 48h.

**Antiviral activity of the polysaccharide fraction of KE**

A polysaccharide fraction was prepared from KE and its effects on the plaque reduction of PRV on Vero cells were analyzed. The plaque reduction assay showed a dose-dependent anti PRV activity of KPF. A 90% inhibition was observed at a concentration of 5mg/ml (Fig. 5).

**DISCUSSION**

Plants are known as an important source of new chemical entities suitable for antiviral drug discovery and development (Hayashi et al., 1997; Xu et al., 1999; Chiang et al., 2002).

This report clearly demonstrated the antiviral activity of KE against PRV in vitro. When the virus and cells were exposed to KE, a concentration dependent inhibition of plaque formation was observed in Vero cells. The IC<sub>50</sub> and CC<sub>50</sub> of KE were 0.24 and 2.4%, respectively. The therapeutic index (CC<sub>50</sub>/IC<sub>50</sub>) of KE was more 10 for PRV. When the virus and cells were exposed to the KE during adsorption, a concentration dependent inhibition of plaque formation was observed. No difference was detected in the plaque formation assays when viruses were treated with KE before adsorption. A 99% reduction in plaque was observed using 1.2% KE. In the attachment assays,
only 25% reduction in attachment was observed using the same concentration of KE. This result made it difficult to explain the 99% reduction in plaque assays. Then we attempted to analyze the expression levels of IE180 of PRV. Suppression of IE180 expression in the presence of KE was observed. PRV expressed a single immediate early protein (IE180) for continuous transcription of early and late genes (Green et al., 1983; Ihara et al., 1983; Wu & Berk, 1988), indicating the IE gene is absolutely necessary for productive lytic infection. These data supported the conjecture that the anti PRV mechanism may be attributed to the prevention of the adsorption of virion to cells and the reduction of IE expression.

KE has various medicinal actions, such as for anti-inflammation, anti-ulcer, antispasmodic, a slight diuretic effect, detoxification, anti-hypertension, anti-hyperglycemia, anti-hyperlipidemia and accelerated wound-healing effects (Shibata et al., 1975; Shibata et al., 1976, 1978; Shibata et al., 1980). Shibata et al. (1978) reported that the potassium of KE has beneficial cardiovascular effects. Moreover, the sugar fractions of molecular weight (MW) less than 5000 da of KE have been shown to stimulate gastric acid secretion (Okabe et al., 1975). Furthermore, the sugar fractions of MW range of 5000 to 10000 da of KE contribute an anti-inflammatory and an antiulcer effect (Shibata et al., 1979).

Polysaccharides have been isolated as active components from different kinds of plants and proved to have anti-viral activity (Hayashi et al., 1996; Hoshino et al., 1998; Huheihel et al., 2002). Polysaccharides have been attributed to contribute to the inhibition of virus adsorption to the cells (Damonte et al., 1996). A polysaccharide fraction of KE (KPF) was examined for its anti PRV effect by the plaque reduction assay. KPF exhibited anti PRV effect with a CC50 of 0.625mg/ml and EC50 of 10mg/ml. The therapeutic index of KPF was more than 16 for PRV. Recently Sakai et al. (2008) reported that anti human cytomegalovirus activity of Sasa albo-margariate have been associated with the tricin. Therefore, KE may include some components for antiviral activity. The antiviral activity of KE in animal models infected with PRV may help clarify the exact role of KE in PRV infection.

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REFERENCES


