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Full Length Research Paper

Two step culture for production of recombinant herpes simplex virus type 2 glycoprotein D in immobilized *Spodoptera frugiperda* cells

Tao Liu[1]*, Hui-jun Dong[2], Ji-feng Liu[3], Wei Zheng[1], Zhi-cheng Huang[1] and Shui-fen Zhu[1]

[1]Microbiology Laboratory, Hangzhou Center for Disease Control and Prevention, Hangzhou 310021, China.
[2]Lunan Pharmaceutical Group Corporation, Linyi 273400, China.
[3]Department of Dermatology, The Third Hospital of Hangzhou, Hangzhou 310009, China.

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Herpes simplex virus type 2 (HSV-2) was the major cause of genital herpes in humans. The HSV-2 glycoprotein D (gD2) had been proved to be a potentially effective vaccine for treatment of genital herpes. The present study was to develop a two step culture to express the recombinant gD2 protein using the immobilized *Spodoptera frugiperda* (Sf9) cells. The first step, Sf9 cells were cultured and harvested in eutrophic medium containing 10% fetal bovine serum. The second step, Sf9 cells were immobilized using silk fibroin hydrogel and cultured in a stirred-tank bioreactor after infection by recombinant baculovirus expressing the full length *gD2* gene. The data shows that the maximum yield of recombinant gD2 protein reached the concentration of 135 mg/L. The results reveal that the immobilized cells and two step culture could extend the expression period and significantly raise the production of the recombinant protein.

Key words: *Spodoptera frugiperda* cells, baculovirus, immobilization, glycoprotein D, silk fibroin hydrogel.

INTRODUCTION

Herpes simplex virus type 2 (HSV-2) is the primary cause of human genital herpes, a common sexually transmitted disease increasing in China. There is a need for development of new therapies or vaccines to reduce transmission of HSV-2. Since glycoprotein gD of HSV-2 (gD2) is a component of the virion envelope which plays an essential role in HSV-2 entering into susceptible mammalian cells, it is a primary vaccine candidate for genital herpes treatment in humans (Pertel et al., 2001). Recently, the utilization of gD2 for prophylactic immunity of genital herpes has been extensively studied and highly anticipated. As large amounts of gD2 are required for such applications, an efficient and economical way of producing gD2 must be established. Although the recombinant full-length or truncated gD2 proteins have been expressed in *E. coli*, yeast and insect cell expression systems, the expression level of recombinant gD2 is not satisfactory for research, clinical and industrial purposes because of the incorrect folding, unsuccessful glycosylation or low production (Fotouhi et al., 2005, 2008; Langenberg et al., 1995; Van Kooij et al., 2002; Landolfi et al., 1993; Watson et al., 1982).

The baculovirus-insect cell system has been extensively used for the expression of recombinant protein for basic research applications, vaccines and diagnostic and therapeutic proteins. It has a disadvantage for the expression system that the specific productivity is decreased as the cell density for infection by baculoviruses is increased (Reuveny et al., 1993). Previous studies have shown that serum in the medium

*Corresponding author. E-mail: liuniu73@yahoo.com.cn. Tel: 086-0571-85063401. Fax: 086-0571-85165007.

Abbreviations: gD2, Glycoprotein D; **Sf9,** *Spodoptera frugiperda*; DO, dissolved oxygen; **MOI,** multiplicity of infection.

played a promoting role on recombinant protein production up to post-infection day 1, but is not essential thereafter (Jardin et al., 2007). Therefore, it is necessary to develop a novel culture method to separate the cell amplification and infection expression.

Silk fibroin is an excellent biomaterial for the formation hydrogel for encapsulating cells since it has good mechanical properties, biocompatibility, and structure network. Silk fibroin has been applied in drug release, tissue engineering and cell immobilization. In this study, Sf9 insect cells were immobilized using silk fibroin to produce recombinant gD2 protein successfully. In addition, a two step culture protocol was investigated. The first step, the insect cells were abundantly proliferated in Grace's insect medium containing serum and glucose. The second step, the insect cells were immobilized and infected with recombinant virus at the proper time in modified Grace's medium (MGM; Grace's medium containing 0.33% lactoalbumin hydrolysate and 0.33% yeastolate) without serum. The two step culture could facilitate downsteam processing and purification of target products and thereby reduces production costs.

MATERIALS AND METHODS

Cell lines, baculovirus and medium

The Bac-to-Bac expression system was purchased from Invitrogen Company (USA). Sf9 cells were gifted from Dr. Chen Yin (Zhejiang Provincial Center for Disease Control and Prevention, China). The Sf9 cells were cultured in Grace's insect medium supplemented with 10% (v/v) fetal bovine serum and glucose (Gibco, USA). The immobilized Sf9 cells were grown in serum-free melanocyte growth medium (MGM). All the cell culture processes were carried out at 27°C. All medium contains 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 g/mL amphotericin. The recombinant baculovirus expressing the full length *gD2* gene of high titer virus (10^8 pfu/mL as determined by plaque assay), named rV-gD2, was obtained and sustained by our lab. The rV-gD2 were propagated by infecting Sf9 cells and harvested from supernatant centrifugated at 1,000 rpm for 5 min to remove cell debris. All virus stocks were stored at 4°C and protected from light to ensure maintenance of titer for gD2 expression.

Cell density was determined by microscopically counting with a Burker-Turk hemocytometer, while cell viability was examined by trypan blue exclusion test.

Shake flask culture of the Sf9 insect cells

Corning 500 mL disposable Erlenmeyer flasks were used with a working volume of 80 mL. After suspension in fresh Grace's insect medium, Sf9 cells were grown for a few days at 27°C in a spinner flask with constant stirring at around 120 rpm. Cells in the exponential growth phase were collected by centrifugation and resuspended in fresh serum-containing medium. The cells were incubated for two days at 27°C on a reciprocal shaker for inoculation. Following removal of the cell suspension, 80 mL of fresh serum-supplemented medium was added to each flask and inoculation on a reciprocal shaker was resumed. The culture medium was replaced completely with fresh serum-supplemented medium every two days.

Immobilized cells culture in bioreactor

The glass bioreactor vessel consisted of a cylindrical body and a rounded bottom. Mixing was performed by an impeller with three blades rotating at the rate of ranging from 0 to 150 rpm in the reactor. A mixed gas of oxygen and nitrogen was introduced from a nozzle at a constant rate set manually according to oxygen demanded. The dissolved oxygen (DO) concentration in the culture medium was controlled at 30% oxygen saturation by automatically changing the composition of the supplied gas based on the reading of a DO sensor. The temperature was kept constant at 27°C by recycling water from a water bath through the tank. The pH was monitored using a pH sensor. But, even without active adjustment, the pH did not change substantially in the course of the cultures. Antifoam agent (Antifoam C Emulsion, Sigma) was added into the bioreactor at the concentration of 0.1 mL/L if necessary.

The process of cells immobilization using silk fibroin was described as the following. Silk cocoons were shattered and then degelatinized twice in boiling water containing 0.5% (w/v) Na_2CO_3 for 1 h. The degelatinized silk fibroin was washed with the double distilled water. The clean silk fibroin was dissolved and boiled in 50% (w/v) $CaCl_2$ at 100°C for 10 min. The cooled silk fibroin solution was dialyzed for 72 h to remove salt using 8 kDa dialysis-membrane. Finally, the silk fibroin was filtered through film (Φ 5 µm, Pharmacia) and subsequently became achroic transparent liquor. For making silk fibroin hydrogel the sterilized silk fibroin remained at 30°C and was mixed with Sf9 cells. The mixture gel subsequently was added into the serum-free MGM medium and stored under 8°C for 30 min. Further, the silk fibroin hydrogel was made into hydrogel beads of about 0.5 mm in diameter (Kim et al., 2004; Matsumoto et al., 2006; Wang et al., 2008).

The time of rV-gD2 addition was designated as post-infection time zero. The hydrogel beads and the culture broth were sampled every day to measure the immobilized cell density and the gD2 protein yield.

Quantitative assay of recombinant gD2

The recombinant baculovirus rV-gD2 infected Sf9 cells when the cell culture reached the later of log phase according to the growth curve (data not shown) for recombinant gD2 expression. The immobilized cells were harvested at 48 to ~72 h post-infection when cells were still mostly viable (between 60 and ~ 90%) and sonicated five times for 10 s by 3 min intervals on ice, using ultrasonicator at 50% power. The lysate was cleared by centrifugation at 10,000 rpm for 30 min at 4°C to remove cell debris. The supernatant was stored at -70°C until protein purification.

The recombinant gD2 protein was determined by enzyme-linked immunosorbent assay (ELISA) (Ausubel et al., 1993). The ELISA standard curve was generated using a series of gD2 standards VTI540 (Meridian life science, USA) from 1.6 to 100 ng/mL (Wang and Fan, 2000). Appropriate wells of 96-well medium binding polystyrene plates (IWAKI, Japan) were coated with serially diluted cell culture samples (100 µl/well). The plates were sealed with a parafilm sheet and incubated in a humidified chamber at 4°C overnight. The plates were allowed to equilibrate to room temperature and were washed three times with freshly prepared phosphate buffered solution containing 0.05% Tween 20 (PBST) using a ELX_{405} auto plate washer (Bio-Tek Instruments Inc., USA). After washing, the plates were blocked with 1% BSA–PBS at room temperature for 2 h. After the plates were washed with PBST three times, mouse anti-gD2 monoclonal antibody C65019M (Meridian life science, USA) were diluted 1:700 in PBST immediately before dispensing into the antigen-coated wells. Hundred microlitres of diluted mouse anti-gD2 monoclonal antibody was then incubated in the cell culture samples coated polystyrene plates at 37°C for 1 h. All incubations in future steps were also performed at 37°C. After

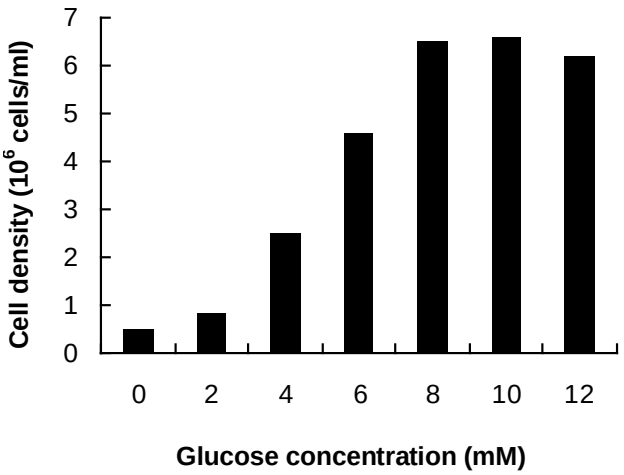

Figure 1. The effect of glucose on Sf9 cell density.

incubation, the wells were washed three times. Each well was then incubated with 100 µl of a 1: 2000 (v/v) dilution of goat anti-mouse I gG conjugated to horseradish peroxidase (Roche, USA) for 45 min. The plates were washed three times. The wells were incubated in the dark with 100 µl of developing solution prepared immediately before use at room temperature for 20 min. The developing reaction in each well was stopped with 40 µl of 2 M H_2SO_4. The optical density at 490 nm was determined for each well with a ELX_{800} Universal Microplate Reader (Bio-Tek instruments Inc., USA). The amounts of recombinant gD2 were calculated from the OD_{490} values that were in the descending portion of the ELISA standard curve for the gD2 standards.

RESULTS

The first step for cell amplification in shake flask

The Sf9 insect cells were cultured in Grace's medium containing 10% fetal bovine serum (FBS) supplemented with limited concentration glucose to obtain the maximum cell density. In the test, the glucose feeding was started at 50 h when the initial quantity of glucose was consumed. The controlled level of glucose was determined depending on the cell density. The results indicate that when the residual concentration of glucose was about 8 mM, the cell density reached 6.5×10^6 cells/mL. A higher concentration of glucose did not give the markable increase of cell density (Figure 1).

The second step for recombinant gD2 protein expression

In this step, the large amount of Sf9 cells obtained at the optimal condition of shake flask culture were immobilized at a density of over 10^6 cells/mL using silk fibroin hydrogel and further made into beads according to the method described in the materials and methods. The immobilized cells were suspended and cultivated using serum-free MGM medium in the 5 L stirred-tank bioreactor. As for the immobilization cell culture, the stirring speed was an important factor to influence the cell growth and oxygen transfer. Therefore the optimal stirring speed was determined in the 5 L bioreactor. The figures showed that the cell density reached the maximum over 8×10^6 cells/mL beads at the stirring speed of 100 rpm. If the stirring speed exceeded 120 rpm, the hydrogel beads were damaged greatly, resulting in decreased viability (Figure 2).

Effect of multiplicity of infection (MOI) on the production of gD2 protein

The effect of multiplicity of infection (MOI) was examined on recombinant gD2 production ranging from 1 to 10. Fed-batch cultures were infected by rV-gD2 after four days when the cell density reached about 6×10^6 cells/mL. The highest yield of recombinant gD2 were obtained at MOI of about 5 with 135 mg/L (Figure 3).

Determination of infection time

Following growth of the immobilized cells to a density of over 10^6 cells/mL beads, a high-titer rV-gD2 was added at MOI of 5. The time of rV-gD2 addition was designated as post-infection time zero. Time of infection is an important factor for obtaining high yield recombinant protein. Previous studies had shown that it was the proper moment of infection when the cell density was going to reach the maximum. In this present study, the non-immobilized cells were cultured in the 5 L bioreactor as the contrast to the culture of immobilized cells. The results show that the immobilized cells grew more slowly than the non-immobilized cells. The optimal infection time should be about 120 to ~130 h (Figure 4).

DISCUSSION

Infection with herpes simplex virus types two remains a prevalent and potentially serious health problem. The gD2 is a primary vaccine candidate for the application in humans. An efficient and economical way of producing gD2 must be established.

The baculovirus expression system has a number of potential benefits, such as high expression level, correct folding and post-translational modification, and production of biologically active proteins for analysis.

Compared with the traditional suspended-cell culture, immobilization of insect cells has some attractive characteristics, such as increasing the biomass, protecting cells from shear force and facilitating the purification of the recombinant protein.

One of the crucial problem in baculovirus-insect cell

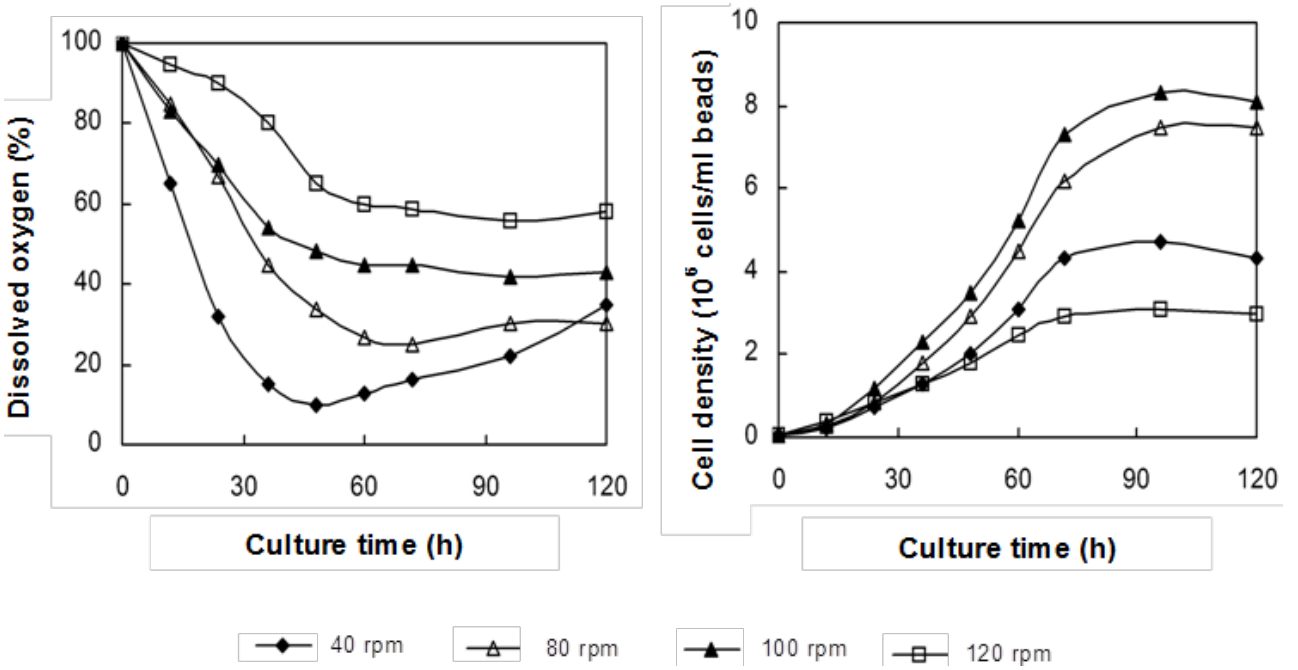

Figure 2. The effect of stirring speed on the DO and Sf9 cell density.

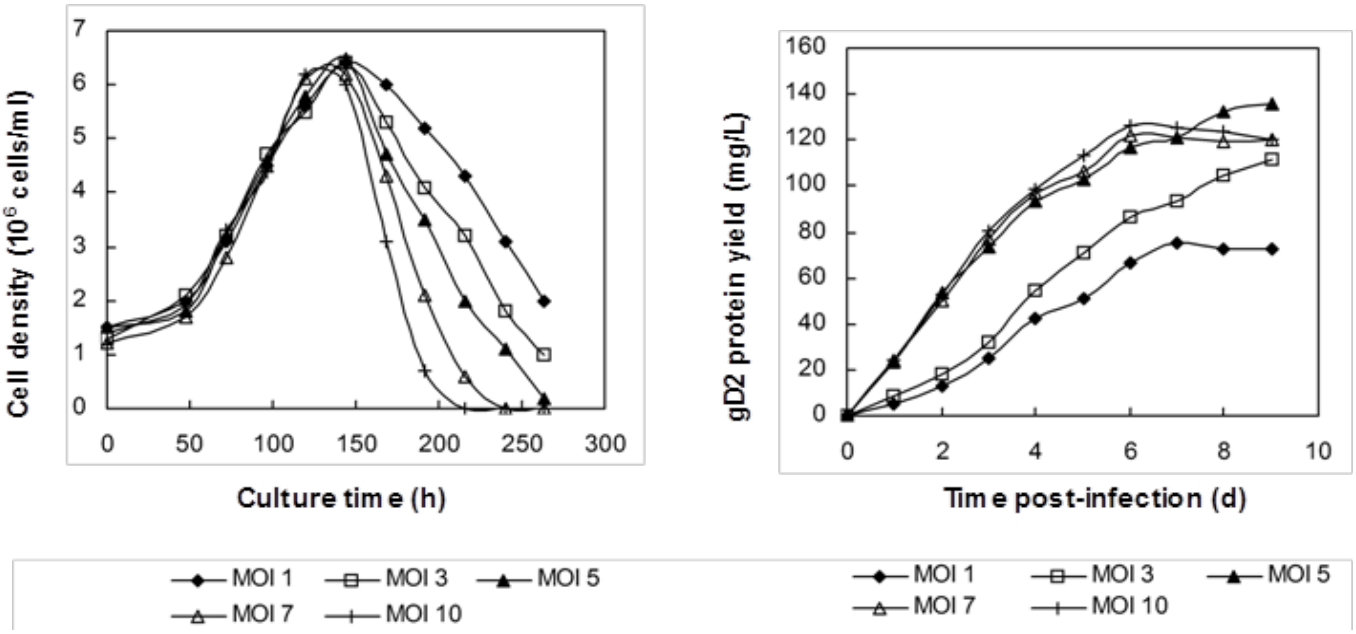

Figure 3. Optimal MOI for Sf9 cells growth and the recombinant gD2 protein production.

expression system was that the interesting protein production was decreased while the cell density for infection was increased. In most cases, the productivity could be improved by supplying the fresh growth medium for cell culture during virus infection. However, the above procedure is impractical on a large scale. In order to obtain higher protein production under the condition of corresponding higher cell density, a combination of cell immobilization and two step culture procedure was developed.

In the present study, a two-step culture of Sf9 cells immobilized within silk fibroin hydrogel beads was carried

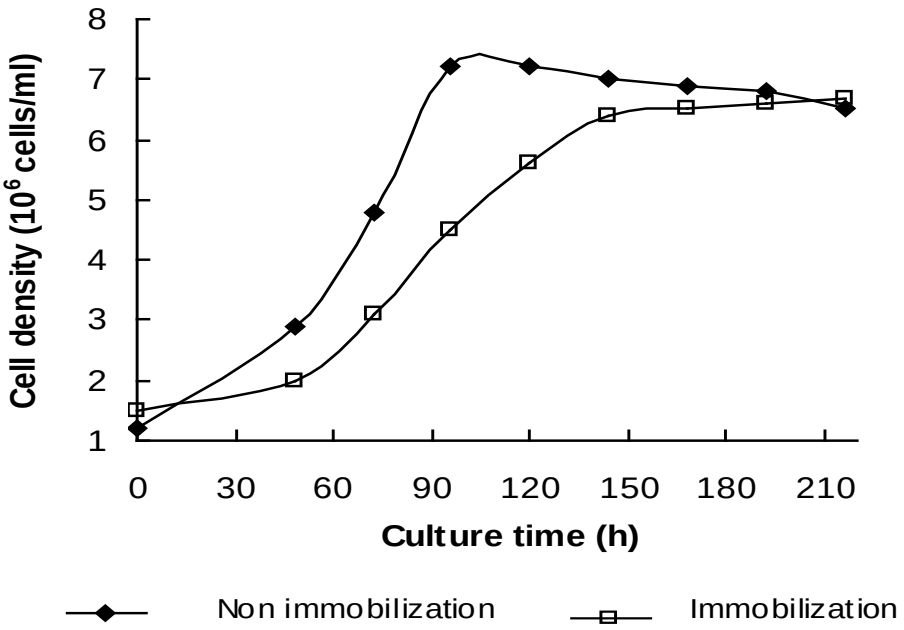

Figure 4. The cells growth process of the immobilized and non-immobilized Sf9 cells.

out in 500 mL shake flasks and a 5 L stirred-tank bioreactor. The first step was to culture cells and the second was to infect cells by rV-gD2 for protein expression. Silk fibroin was a suitable bio-material to be used as immobilization vector (Matsumoto et al., 2006). In the first step of shake flask culture, Sf9 cells were amplified in Grace's insect medium supplemented with fetal bovine serum and glucose, and which could increase the cell density to 10^6 cells/mL. During the baculovirus infection step, the infection time was determined at the logarithmic growth phase of immobilized Sf9 cells. Recombinant protein production by densely immobilized cells in serum-free MGM medium could simplify separation and purification processes and reduce the manufacturing costs of cellular products. In this research, the dissolved oxygen concentration was maintained at 30% oxygen saturation in the bioreactor. Maybe, excessive DO conditions can bring the formation of nascent oxygen, superoxide and peroxide which destroy cellular components and then destroy the cell growth. In our previous studies, the protein production by immobilized Sf9 insect cells was significantly depressed when the DO was below than 30%. The DO level was higher than the culture of non-immobilized cells since the immobilization limited the transfer of oxygen. Specially, the Bac-to-Bac expression system was chosen since it is a valid and multipurpose system for high production of recombinant protein. The specific gD2 protein productivity obtained by immobilized cell in bioreactor culture was higher than that by non-immobilized cell culture.

Due to the fact that glucose, stirring speed, MOI and infection time are the most essential factors affecting Sf9 cells growth, these factors have been investigated with the aim of increasing the final cell density for high protein production. In this study, the concentrations of glucose, stirring speed, MOI and infection time were optimized in cell culture. Higher cell density could be achieved at the conditions of 8 mM glucose. In contrast, lower concentrations of glucose could not provide enough carbohydrate for cell metabolism and propagation, and higher concentrations of glucose were not beneficial to cell growth due to the production of extra lactic acid or ammonia. Although the higher stirring speed was a crucial factor for increasing the concentration of the dissolved oxygen and nutrition transfer in the immobilized cell culture, the shear force of agitator at high speed would damage the gel beads and cells. Higher cell density could be achieved at the stirring speed of 100 rpm, infection time of 120 to 130 h and MOI of 5.

At the optimal conditions, the yield of recombinant gD2 protein was up to 135 mg/L cell culture, to our knowledge this is the first report of the combination of immobilization and two-step culture for the recombinant gD2 protein expression in bioreactor.

In the further research, we will optimize the culture conditions of the two step culture to separate the medium from the bioreactor and replace with other medium in order to obtain higher protein productivity.

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