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Recombinant protein production by the baculovirus–insect cell system in basal media without serum supplementation

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Abstract

The production of β-galactosidase by Sf9 cells infected with recombinant *Autographa californica* nucleopolyhedrovirus (AcNPV) was investigated in shake-flask culture using two serum-free basal media: Grace's medium and TNM-FH (Grace's medium supplemented with lactalbumin hydrolysate and yeast extract). At the time of infection, cells grown in serum-supplemented TNM-FH were transferred into fresh basal media without adaptation. The absence of serum depressed the β-galactosidase yield considerably in Grace's medium, but to a much lesser extent in TNM-FH, where it reached around 2/3 of the level obtained in TNM-FH supplemented with 10% fetal bovine serum (FBS). While both lactalbumin hydrolysate and yeast extract promoted β-galactosidase production, their removal by medium replacement on post-infection day 1 gave a β-galactosidase yield nearly equal to that obtained in their continuous presence. Supplementation of basal media with phosphatidic acid (PA) from egg yolk lecithin, which has been shown to enhance cell growth and recombinant protein production in serum-free culture of Chinese hamster ovary (CHO) cells, was also effective in increasing β-galactosidase yield. Elevating the multiplicity of infection (MOI) from 2 to 10 plaque-forming units per cell (pfu/cell) also resulted in an increase in product yield. These results provide information important to the development of cost-effective serum-free culture technology for use in large-scale production of recombinant proteins by the baculovirus–insect cell system.

Introduction

The baculovirus–insect cell system has been shown to be an excellent expression system capable of providing large quantities of biologically active recombinant proteins. It involves the construction of a recombinant nucleopolyhedrovirus containing the foreign gene of interest under the control of the very late polyhedrin promoter, which is exceptionally strong. Upon infection with the recombinant baculovirus, cultured insect cells often express very high levels of recombinant protein through post-translational processing and modification of higher eukaryotes. The highly restricted host range of the baculovirus makes this expression system safe. Hence, the baculovirus–insect cell system is increasingly used not only in the expression of recombinant proteins for basic research applications but also in the production of a wide variety of valuable proteins including therapeutic proteins and vaccines (Silberklang et al. 1995; Yang et al. 1996; Cruz et al. 1999).

Insect cells have been widely cultured in basal media supplemented with vertebrate serum, in particular with a concentration of 5% to 20% of fetal bovine serum (FBS), which has been shown to support cell growth, baculovirus infection, and recombinant protein production (Wu et al. 1989). The use of serum, however, has a number of disadvantages, including high cost, variable lot-to-lot performance, difficulties in downstream processing and purification of target proteins, and potential biohazards arising from possible pathogenic contaminants such as mycoplasmas, viruses, and proteinaceous infectious particles (prions). These problems have led to the development of serum-free media for use in insect cell culture. Serum-free media are currently available commercially, but more cost-effective medium formulations are still required for large-scale production of recombinant proteins (Maiorella et al. 1988; Jesionowski and Ataai 1997; Donaldson and Shuler 1998).

In the present study, with the aim of developing an effective serum-free culture technology using the baculovirus–insect cell system, we investigated the production of recombinant protein by baculovirus-infected insect cells in basal media without serum supplementation. TNM-FH was selected as one of the basal media because the serum-containing medium is widely used to maintain insect cell cultures. Degradation of recombinant proteins by proteases derived from the virus-infected cells occurs frequently in serum-free culture (Silberklang et al. 1995; Cruz et al. 1999; Yamaji et al. 2003). β-Galactosidase however is reported to be relatively stable against the proteases produced in the baculovirus–insect cell system (Gotoh et al. 2001; Yamaji et al. 2003), and was therefore employed as the model recombinant protein. Our results showed that serum-free TNM-FH supported a considerable level of β-galactosidase production by Sf9 cells infected with a recombinant baculovirus.

Materials and methods

Cell line and baculovirus

The insect cell line used was Sf9 (Invitrogen, Carlsbad, CA, USA) derived from the pupal ovarian tissue of the fall army worm, *Spodoptera frugiperda*. The recombinant baculovirus used was *Autographa californica* nucleopolyhedrovirus (AcNPV) containing the *Escherichia coli* β-galactosidase gene inserted downstream of the polyhedrin promoter instead of the polyhedrin gene (Invitrogen). The virus stock solution titer was determined by plaque assay as described previously (Yamaji et al. 1999).

Media and additives

Sf9 cells were maintained at 27 °C in T-flasks in a non-humidified incubator. The culture medium used for routine maintenance was TNM-FH, consisting of 51.19 g l^{-1} TNM-FH (Sigma Chemical, St. Louis, MO, USA) and 0.35 g l^{-1} $NaHCO_3$, with addition of 10 mg l^{-1} gentamicin sulfate (Invitrogen) and 1 g l^{-1} Pluronic F-68 [block co-polymer glycol of poly(oxyethylene)-poly(oxypropylene)-poly(oxyethylene), Sigma], supplemented with 10% FBS (Invitrogen). TNM-FH is a modification of Grace's medium by supplementation with 3.33 g l^{-1} lactalbumin hydrolysate and 3.33 g l^{-1} yeast extract, and was originally formulated to establish primary culture of *Trichoplusia ni* cells (Hink 1970). In the shake-flask culture, serum-free TNM-FH and Grace's medium [44.5 g l^{-1} Grace's medium (Sigma), 0.35 g l^{-1} $NaHCO_3$, 10 mg l^{-1} gentamicin sulfate, and 1 g l^{-1} Pluronic F-68] were employed as well as 10% FBS-supplemented TNM-FH and Grace's medium. In some cases, these serum-free basal media were supplemented with either lipid mixture (Sigma) to give final concentrations of 4.5 mg l^{-1} cholesterol; 10 mg l^{-1} cod liver oil fatty acids (methyl esters); 25 mg l^{-1} Tween 80 (polyoxyethylenesorbitan monooleate); and 2 mg l^{-1} D-α-tocopherol acetate; or 10 mg l^{-1} phosphatidic acid (PA) from egg yolk lecithin (Sigma) prepared with Tween 80 at a Tween/PA ratio (w/w) of 0.1 as described previously (Sakai et al. 2001). Grace's medium supplemented with either 3.33 g l^{-1} lactalbumin hydrolysate or 3.33 g l^{-1} yeast extract was also tested.

Shake-flask culture

Sf9 cells were dislodged from T-flasks by streaming medium over the adherent cells with a

pipette. After suspension in fresh serum-supplemented TNM-FH, cells were grown for a few days at 27 °C in a spinner flask with constant stirring at around 90 rpm. To evaluate the growth of uninfected cells in basal media, cells in the exponential growth phase were collected by centrifugation and resuspended at 2–3 $\times$ 10^5 cells/cm^3 in 15 ml of fresh medium in screw-capped 100-ml Erlenmeyer flasks. The cells in the flasks were cultivated at 27 °C on a reciprocal shaker (90 oscillations/min, amplitude 30 mm). Every day, 200 μl of cell suspension was removed from each flask to measure cell density, which was determined by microscopically counting the number of cells with a Bürker-Türk hemocytometer, and cell viability, which was judged by dye exclusion using trypan blue.

To examine β-galactosidase production by virus-infected cells, cells collected as described above were resuspended at approximately 1 $\times$ 10^6 cells/cm^3 in 15 ml of fresh serum-containing or serum-free media in screw-capped 100-ml Erlenmeyer flasks. High-titer viral stock solution was added to give a multiplicity of infection (MOI) of 2 or 10 plaque-forming units per cell (pfu/cell). The cells in the flasks were then cultivated on a reciprocal shaker in a manner similar to the above. Every day, 400μl of the cell suspension was removed from each flask to measure cell density and β-galactosidase. Aliquots of the cell suspension were stored at −20 °C for β-galactosidase activity assay.

Assay for β-galactosidase activity

To release intracellular β-galactosidase, 450 μl of 0.556% Triton X-100 (polyethylene glycol mono-*p*-isooctylphenyl ether) in Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, and 0.05 M 2-mercaptoethanol, pH 7.0) was added to 50-μl samples of the cell suspension and the preparation diluted appropriately (1:7 or 1:9) in Z buffer. β-Galactosidase activity was determined by enzymatic hydrolysis of *o*-nitrophenyl-β-D-galactoside (ONPG) to *o*-nitrophenol and galactose as previously described (Yamaji et al. 1999). One unit (U) of β-galactosidase activity is defined as the amount of enzyme required to hydrolyze 1 μmol of ONPG to *o*-nitrophenol per minute at 28 °C and pH 7.0.

Results and discussion

Cell growth in basal media

Grace's medium and TNM-FH were first compared for uninfected Sf9 cell growth under serum-containing or serum-free conditions (Figure 1). In 10% FBS-supplemented Grace's and TNM-FH media, the maximum cell density achieved was 2.0 $\times$ 10^6 and 2.7 $\times$ 10^6 cells/cm^3, respectively. Grace's medium without serum supplementation, on the other hand, did not support cell growth (Figure 1a). In the case of serum-free TNM-FH (Figure 1b), cells grew at a slower rate and reached

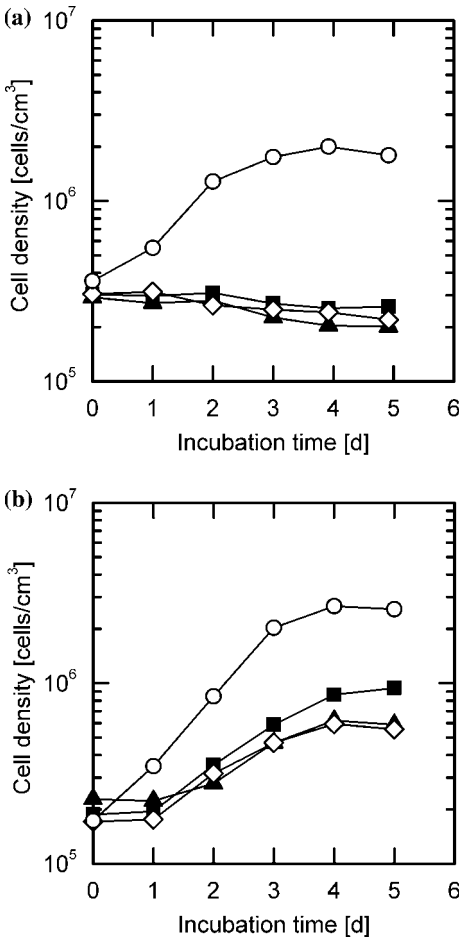

Figure 1. Growth of Sf9 cells in shake-flask culture with (a) Grace's medium and (b) TNM-FH. Symbols: ○, medium supplemented with 10% fetal bovine serum (FBS); ■, medium supplemented with lipid mixture; ▲, medium supplemented with 10 mg l^{-1} phosphatidic acid (PA) from egg yolk lecithin; ◇, medium without supplementation.

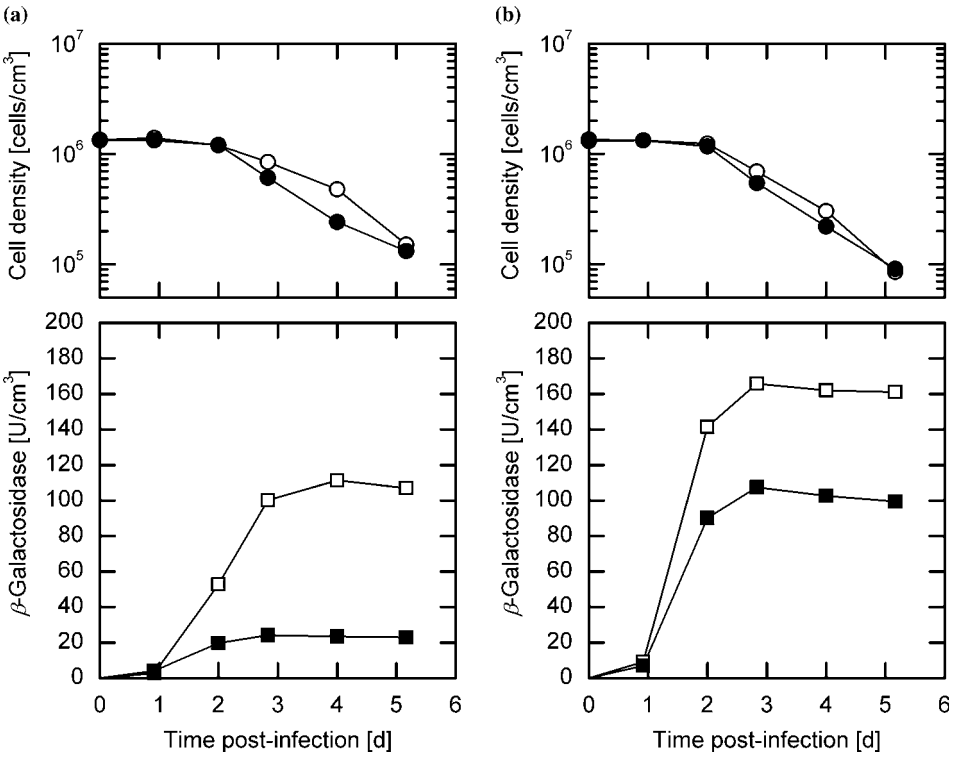

Figure 2. Production of β-galactosidase by recombinant baculovirus-infected Sf9 cells in shake-flask culture with (a) Grace's medium and (b) TNM-FH. Cells were infected at a multiplicity of infection (MOI) of 2 plaque-forming units (pfu)/cell. Open symbols: medium supplemented with 10% FBS; filled symbols: medium without supplementation.

6×10^5 cells/cm^3, or 22% of the maximum cell density obtained in the serum-containing medium. Admixture of lipids, which are reported to stimulate serum-free growth of insect cells (Maiorella et al. 1988), did not improve cell growth in Grace's medium, but did in TNM-FH. Although PA has been shown to promote the growth of Chinese hamster ovary (CHO) cells in serum-free culture (Sakai et al. 1999; 2002), supplementation of Grace's medium and TNM-FH with PA from egg yolk lecithin was not effective in increasing the density of Sf9 cells. In the present study, Sf9 cells were grown after direct transfer from TNM-FH containing 10% FBS into serum-free basal media without adaptation, which may have resulted in poor cell-growth.

Protein production in basal media

Recombinant β-galactosidase production by baculovirus-infected Sf9 cells in the serum-free basal media was then compared with that in the serum-containing media (Figure 2). Since basal media did not support good growth of Sf9 cells under serum-free conditions, cells grown in serum-supplemented TNM-FH were collected by centrifugation and suspended in fresh serum-containing or serum-free media at the time of infection. In both serum-supplemented and serum-free media, the viable cell density remained nearly constant during the initial culture period (0–2 d). After 3 days, the viable cell density rapidly decreased. These results may indicate that almost all the cells were synchronously infected with the initially added baculovirus. Abundant β-galactosidase production was observed on post-infection day 2 and the concentration reached a plateau around day 3. With 10% FBS-supplemented Grace's medium and TNM-FH, the β-galactosidase yield was 110 and 165 U/cm^3, respectively. Under serum-containing conditions, a higher product yield was achieved in TNM-FH than in Grace's medium. In serum-free Grace's medium, the β-galactosidase yield was 25 U/cm^3, only 1/5 of that obtained in the serum-supplemented medium. In serum-free TNM-FH,

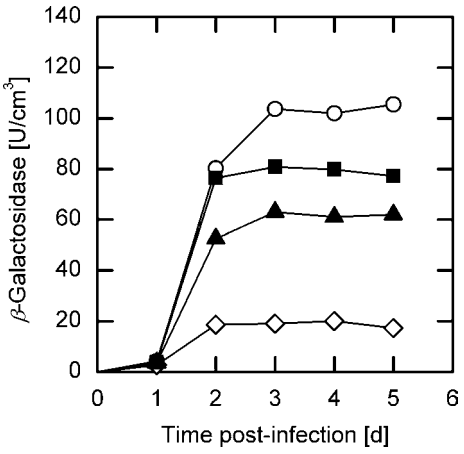

Figure 3. Effect of lactalbumin hydrolysate and yeast extract on β-galactosidase production. Cells were infected at an MOI of 2 pfu/cell. Symbols: ○, TNM-FH; ■, Grace's medium supplemented with 3.33 g l^{-1} yeast extract; ▲, Grace's medium supplemented with 3.33 g l^{-1} lactalbumin hydrolysate; ◇, Grace's medium.

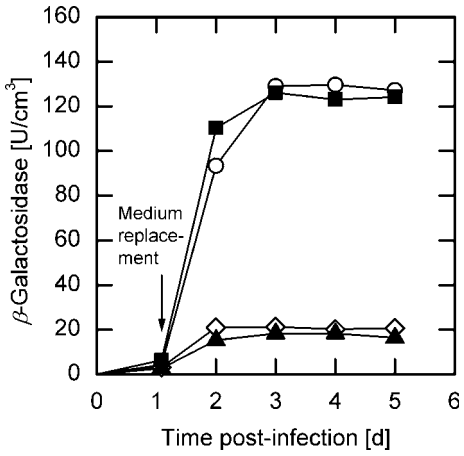

Figure 4. Effect on β-galactosidase production of medium cross-replacement with TNM-FH and Grace's medium on post-infection day 1. Cells were infected at an MOI of 2 pfu/cell. Symbols: ◯, TNM-FH replaced with fresh TNM-FH; ■, TNM-FH replaced with fresh Grace's medium; ▲, Grace's medium replaced with fresh TNM-FH; ◇, Grace's medium replaced with fresh Grace's medium.

in contrast, a relatively high β-galactosidase yield, around 2/3 of that obtained with serum-supplemented TNM-FH, was achieved. These results indicate that lactalbumin hydrolysate and/or yeast extract promote recombinant protein production by AcNPV-infected Sf9 cells under both serum-containing and serum-free conditions.

To find out which of lactalbumin hydrolysate or yeast extract is more effective in stimulating recombinant protein production, their effects on β-galactosidase production in Grace's medium were investigated. As can be seen from Figure 3, both had a promoting effect on β-galactosidase production, with yeast extract the more effective. They were also found to have an additive effect on recombinant protein production. Lactalbumin hydrolysate is a source of small peptides and amino acids, and yeast extract a source of amino acids, vitamins, nucleotides, and other nutrients. Since both are chemically undefined and may show lot-to-lot variation, the actual compounds responsible for promoting recombinant protein production need to be identified.

Temporal effect of lactalbumin hydrolysate and yeast extract

As can be seen from Figures 2 and 3, β-galactosidase production in large quantities was attained on post-infection day 2 following a latent period of one day. To further investigate the effects of lactalbumin hydrolysate and yeast extract on protein production, shake-flask cultures were performed with medium replacement through centrifugation on day 1 (Figure 4). When lactalbumin hydrolysate and yeast extract were removed by replacing TNM-FH with Grace's medium, virus-infected cells went on to produce β-galactosidase in a similar manner to those in a control culture in which TNM-FH was replaced with fresh TNM-FH, and with nearly equal product yield. A comparable β-galactosidase yield was also obtained in a culture using TNM-FH without medium replacement (data not shown). In contrast, when lactalbumin hydrolysate and yeast extract were added by medium replacement on day 1, the β-galactosidase yield remained as low as in a control culture in which lactalbumin hydrolysate and yeast extract were absent throughout. A similar pattern was observed in shake-flask cultures with medium cross-replacement with Grace's medium and Grace's medium supplemented with either lactalbumin hydrolysate or yeast extract (data not shown). When TNM-FH was replaced with Grace's medium before 24 h post-infection, the earlier the medium was replaced, the lower the β-galactosidase yield became (data not shown). These results clearly show that lactalbumin

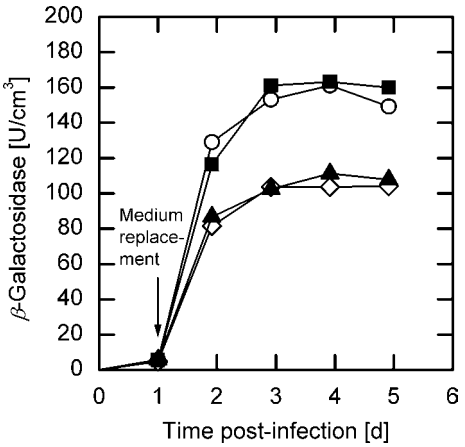

Figure 5. Effect on β-galactosidase production of medium cross-replacement on post-infection day 1 with serum-free TNM-FH and TNM-FH supplemented with 10% FBS. Cells were infected at an MOI of 2 pfu/cell. Symbols: ○, serum-supplemented TNM-FH replaced with fresh serum-supplemented TNM-FH; ■, serum-supplemented TNM-FH replaced with fresh serum-free TNM-FH; ▲, serum-free TNM-FH replaced with fresh serum-supplemented TNM-FH; ◇, serum-free TNM-FH replaced with fresh serum-free TNM-FH.

hydrolysate and yeast extract exert a promoting effect on recombinant protein production up to post-infection day 1, but are not essential thereafter. Figure 5 compares the effect of medium replacement with serum-supplemented and serum-free TNM-FH on β-galactosidase production. FBS was also found to be essential up to day 1 in promoting β-galactosidase production. These results may indicate that lactalbumin hydrolysate, yeast extract, and FBS stimulate recombinant protein production through similar mechanisms up to the very late stages of the virus infection cycle, at which recombinant proteins are abundantly produced instead of polyhedrin.

The above finding that lactalbumin hydrolysate, yeast extract, and FBS are not essential after post-infection day 1 may offer a promising method of recombinant protein production: a two-step culture in which cells are first grown and virus-infected in a serum-containing medium and recombinant protein is then produced in a serum-free basal medium after medium replacement on post-infection day 1. In this culture, recombinant protein production is achieved in a protein-free medium, which can facilitate downstream processing and purification of target proteins and thereby reduce production costs. This applies to the production of intracellular

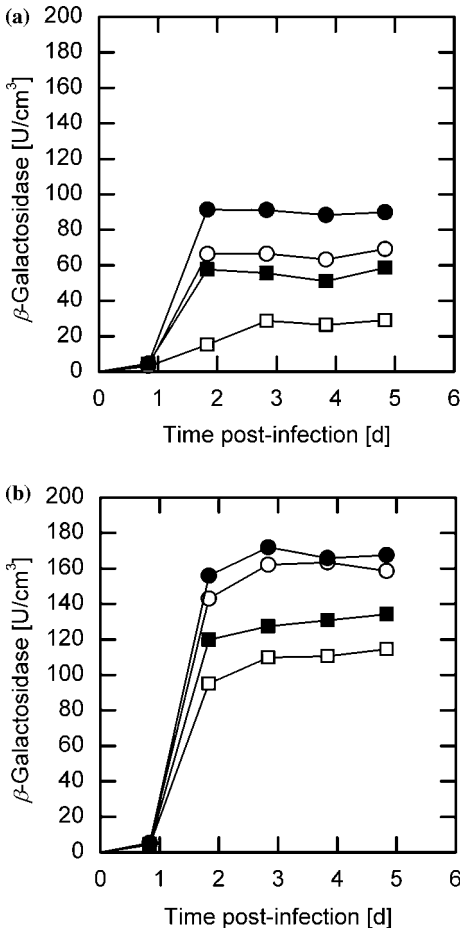

Figure 6. Effect of MOI on β-galactosidase production in (a) Grace's medium and (b) TNM-FH. Symbols: ●, medium supplemented with 10% FBS, MOI = 10 pfu/cell; ○, medium supplemented with 10% FBS, MOI = 2; ■ medium without supplementation, MOI = 10; □ medium without supplementation, MOI = 2.

proteins including β-galactosidase by the baculovirus–insect cell system since the proteins can be released into the culture medium, resulting from the loss of cell membrane integrity because of the lytic nature of the virus infection process (Licari and Bailey 1991; Yamaji et al. 2000; Yamaji et al. 2003). The two-step culture however requires medium replacement in the course of the culture, which may be considered difficult on a large scale. Medium replacement can be carried out simply and easily through immobilization within porous biomass support particles (Yamaji et al. 2000). The two-step culture using this immobilization technique is currently under investigation.

Effect of MOI and PA

As recombinant protein production in the baculovirus–insect cell system is affected by MOI, an examination was undertaken of its effect on β-galactosidase production in basal media (Figure 6). In both serum-supplemented and serum-free Grace's media, β-galactosidase yield increased with increase in MOI from 2 to 10 pfu/cell (Figure 6a), but in TNM-FH supplemented with 10% FBS, as reported previously in high MOI infections (Yamaji et al. 1999), nearly equal product yields were obtained irrespective of MOI; in serum-free TNM-FH, meanwhile, baculovirus infection at the higher MOI value caused an increase in β-galactosidase yield (Figure 6b). These results may indicate a possibility that AcNPV has lower infectivity to Sf9 cells in serum-free basal media than in serum-containing media, while even in the former, all the cells appear to be infected with the initially added virus at an MOI of 2 pfu/cell (Figure 2). Further investigation is required to elucidate the mechanism of increased recombinant protein production at high MOI values.

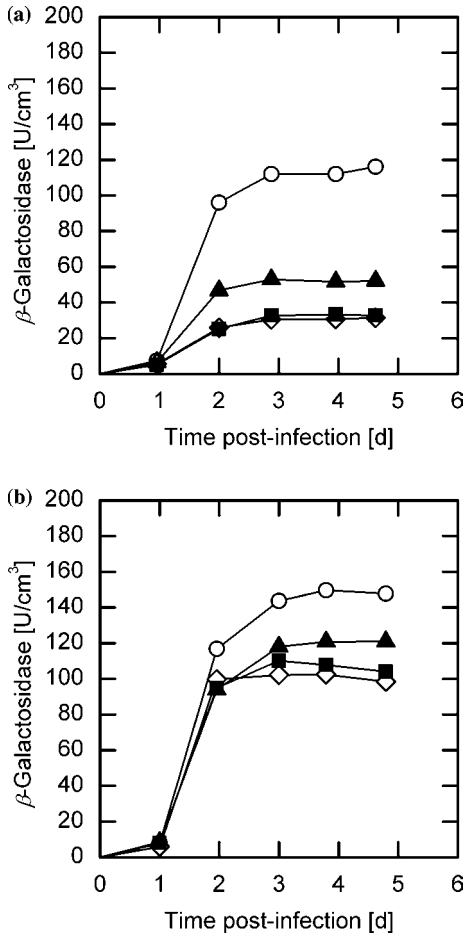

Figure 7. Effect of PA on β-galactosidase production in (a) Grace's medium and (b) TNM-FH. Cells were infected at an MOI of 2 pfu/cell. Symbols as in Figure 1.

Finally, supplementation of basal media with lipids or PA from egg yolk lecithin was studied (Figure 7). Although the lipid mixture stimulated the growth of uninfected cells in serum-free TNM-FH (Figure 1b), it did not affect β-galactosidase production under serum-free conditions in either Grace's medium or TNM-FH. In contrast, PA was effective in increasing β-galactosidase yield in both Grace's medium and TNM-FH. Culture experiments with medium replacement showed that, like lactalbumin hydrolysate, yeast extract, and FBS, PA was essential up to day 1 in stimulating β-galactosidase production (data not shown). While the mechanism by which it promotes recombinant protein production remains unknown, this finding identifies PA as a promising supplement for use in low-cost serum-free medium. Taken together, the results obtained in the study provide information important to the development of low-cost serum-free culture technology for use in large-scale production of recombinant proteins by the baculovirus–insect cell system.

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