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Identification of bioactive yeastolate fractions responsible for insect cell growth and baculovirus production

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Abstract

Yeastolate is an efficient serum replacement to fully support insect cell growth and baculovirus replication in the insect cell/baculovirus expression system. However, yeastolate is an undefined complex substance and therefore subject to batch-to-batch variation, which often causes inconsistent cell growth and productivity. Therefore, the present study aims to identify yeastolate components that promote insect cell growth and baculovirus production. Gel filtration chromatography was used to determine the molecular weight profiles of yeastolate and fresh/spent culture media during Sf-21 insect cell growth and infection. By comparing the differences among these molecular weight profiles, yeastolate was fractionated into five different molecular weight ranges of fractions. Yeastolate fraction with molecular weight below 1 kD was found to contain bioactive components responsible for the cell growth, but the ingredients essential for baculovirus production were localized in yeastolate fraction between 1 to 3 kD. These results indicate that nutrient utilization was significantly different during the cell growth and infection. The present study provides a high-resolution guideline for further identification of specific bioactive ingredients in yeastolate to develop fully chemically-defined serum-free media for the insect cell/baculovirus system.

Keywords: Insect cell, Baculovirus, Polyhedra, Serum-free medium, Yeastolate

Introduction

Increased interest in the use of the insect cell/baculovirus system for the production of recombinant proteins and viral pesticides has boosted urgent demands on developing efficient culture media to enhance the cell growth and product yield in a cost-efficient way. In addition to glucose, salts, amino acids and vitamins, typical insect cell culture media are supplemented with 10% high-cost serum that contains complex and uncharacteristic natural ingredients essential for the cell growth and baculovirus replication. However, highly variable serum products derived from animals are subject to lot-to-lot variation that often causes inconsistent cell growth and productivity. Therefore, there have been extensive studies dedicated to searching for low-cost and chemically-defined alternative serum substitutes in the past decades (Hink 1991; Reuveny et al. 1992; Schlaeger 1996; Vaughn and Fan 1997). Generally, serum serves as a source of lipids (Goodwin 1991) and growth factors such as hormones and oligopeptides (Schlaeger 1996). Accordingly, any serum replacement ought to mimic this formulation. In the present, protein hydrolysates such as yeastolate (or yeast extract) along with several lipid mixtures have been found to efficiently replace serum to support insect cell growth and baculovirus production.

Yeastolate is produced from aqueous extract of baker's or brewer's yeast via autolysis and contains chemically-undefined complex ingredients such as amino acids, oligopeptides, vitamin, and nucleic acids (Reuveny et al. 1993; Schlaeger 1996; Wu and Lee 1998). Clearly, some components within yeastolate are essential for insect cell growth and recombinant protein production (Ferrance et al. 1993). The addition of yeastolate during high insect cell density would further stimulate the cell growth (Drews et al. 1995; Lee and Park 1994; Wu and Lee 1998), but feeding highly concentrated yeastolate (e.g. 16 g/L) inhibited cell growth (Drews et al. 1995). Moreover, insect cell growth and recombinant protein production can be significantly enhanced by feeding glucose, glutamine, lipids and yeastolate (Nguyen et al. 1993); partly increased by addition of glucose, glutamine and yeastolate (Nguyen et al. 1993; Reuveny et al. 1993); but not by simple replenishment of glucose, glutamine, and/or lipids (Caron et al. 1990; Nguyen et al. 1993; Wu et al. 1993). Apparently, yeastolate along with lipids can provide most comprehensive counterpart ingredients corresponding to serum, but yeastolate plays more crucial role than lipids in supporting insect cell growth and recombinant protein production. The quality of yeastolate also varies from batch to batch due to inconsistent fermentation and downstream processes (Zhang et al. 2003). Therefore, much effort has been put into identifying growth-promoting and productivity-enhancing ingredients in yeastolate in

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an attempt to replace yeastolate with the more defined active substances. However, screening several defined components such as vitamin concentrate or nucleic fractions (purines and pyrimidines) to replace yeastolate was not successful (Reuveny et al. 1993; Shen et al. 2007; Wu and Lee 1998). A recent study using sequential ethanol precipitation for yeastolate fractionation was reported to identify potentially active yeastolate fractions that promote Sf-9 cell growth and recombinant protein production (Shen et al. 2007). However, poor separation resolution by ethanol precipitation resulted in serious overlapping among the yeastolate fractions, which complicates the further characterization and evaluation of active ingredients in yeastolate. Hence, more high-resolution isolation studies are needed to identify individual bioactive components in yeastolate.

In this work, the effects of yeastolate and medium osmolarity on Sf-21 insect cell growth and polyhedra production was investigated to determine the optimal yeastolate concentration and osmolarity in a serum-free medium. Subsequently, the molecular distribution of yeastolate was determined by gel filtration chromatography to provide a guideline for yeastolate fractionation strategy. The effects of each fraction on the cell growth and productivity were evaluated to identify bioactive fractions that are responsible for the cell growth and polyhedra production. Finally, two yeastolate fractions were identified to possess the complete activity of yeastolate for the cell growth and polyhedra production.

Materials and Methods

Cell line, culture medium and baculovirus

The *Spodoptera frugiperda* IPLB-Sf-21AE cell line (designated as Sf-21) was maintained at 27.5 °C in a 125-ml disposable shaker flask (Corning) with 15 ml (working volume) of IBL-10 serum-free and glutamine-free medium (Vaughn and Fan 1997) at 150 rpm. The medium is a modified IPL-41 medium (JRH Biosciences), in which glucose (10 g/L) is the only carbohydrate source and glutamine and organic acids are completely omitted. The fetal bovine serum was replaced with 10 g/L yeastolate (Difco, Detroit, MI) and 0.1% (v/v) 1000-fold concentrated lipid mixture (Sigma, St. Louis, MO). The cells were routinely subcultured every three days at a seed density of 0.4×10^6 cells/ml. Cell counts were determined with a hemocytometer and viable cells were identified by trypan blue exclusion. Cell cultures were passed at least three times in any experiments performed in the study. For infection experiments, the cells at a density of 2×10^6 cells/ml were infected by wild-type *Autographa californica* nuclear polyhedrosis baculovirus with a MOI of 1 $TCID_{50}$/cell to ensure synchronous infection. Polyhedra count was determined with a hemocytometer from culture samples at 5 days postinfection. All experiments were performed in triplicate and all experimental data reported, hence, represent the mean of triplicate estimations ± standard deviation.

Test media

Different test media were prepared by varying the initial concentrations of yeastolate in basal IBL-10 medium at osmolarity between 340 and 350 mOsm/kg. The Sf-21 cells were grown in each test medium at least 3 passages prior to sample acquisition, and each experiment on the effects of different test media on cell growth and product yield was performed in triplicate to ensure experimental consistency and reproducibility.

Determination of molecular weight distributions

Gel filtration chromatography was used to determine the molecular weight distributions of complex nutrients such as yeastolate and fresh/spent culture media. Due to their unknown molecular weight profiles, a column with wide fractionation range, Hiprep Sephacryl S-200 HR gel filtration column (Pharmacia Biotech, NJ), was selected to ensure the complete recovery of all the ingredients in the complex nutrients. To obtain a good resolution, a small volume (1 ml) of sample was injected into the column and eluted with a 0.1 M phosphate buffer adjusted to pH 6.3 at a flow rate of 0.7 ml/min. Eluents were collected and scanned at 280 nm by a Nanodrop spectrometer (Thermo Scientific, Wilmington, DE). Molecular weight standards for gel filtration chromatography were purchased from Sigma. The void volume (v_o) of the column was determined as the elution time of blue dextran.

Test media preparation of yeastolate fractions

Five yeastolate fractions with different molecular weight ranges were collected from 10 g/L yeastolate using membrane ultrafiltraton and dialysis tubing. The five molecular weight ranges are (1) < 10 kD, (2) 1-10 kD, (3) < 5 kD, (4) < 3 kD, and (5) < 1 kD. The 10 kD cutoff ultrafiltraton tubes were purchased from Millipore (Billerica, MA). 3 kD cutoff membrane tubes were obtained from Amicon (Danvers, MA). The < 5 and < 1 kD fractions were collected by Molecular/Por® 5 kD ultrafiltraton membrane and Spectra/Por® 1 kD dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA), respectively. These collected yeastolate fractions were separately added into the basal IBL-10 media with 0.1% lipids. These newly prepared test media were finally adjusted to osmolarity between 340 and 350mOsm/kg with mannitol.

Analytical

Glucose and lactate concentrations were determined with an YSI Model 2700 glucose analyzer (Yellow Springs Instrument, Yellow Springs, OH). Osmolarity were measured with an osmometer (Advanced Instrument Inc., MA).

Results and Discussion

Effects of yeastolate and osmolarity on Sf-21 cell growth

As reported in the literature, feeding highly concentrated yeastolate at high cell density inhibited insect cell growth (Drews et al. 1995). This study was based on direct feeding of yeastolate, in which the osmolarity change in the culture medium was not taken into consideration. It is possible that the inhibition effect was due to high osmolarity resulting from feeding highly concentrated yeastolate. This assumption can be verified by varying the initial yeastolate concentration and medium osmolarity in Sf-21 cell cultures to investigate how the cell growth is affected by yeastolate and osmolarity at different initial levels. Figure 1A shows the effects of yeastolate and osmolarity on the cell growth in five different concentrations of yeastolate and two different levels of medium osmolarity at 340-350 mOsm/kg (normal) and 390-400 mOsm/kg (high). No cell growth was observed in the yeastolate-free culture, indicating that yeastolate clearly provides vital components that are essential for the cell growth. Higher initial concentration of yeastolate led to a higher maximum cell density at normal medium osmolarity. The saturation of maximum cell density (14.4×10^6 cells/ml) in the culture with 20 g/L of yeastolate was due to glucose depletion in the culture medium (Figure 1A). On the other hand, high initial medium osmolarity resulted in much lower maximum cell densities in cultures with all levels of yeastolate, and meanwhile nutrients such as glucose (Figure 1A) and amino acids (data not shown) still remained ample. Very likely, the reported cell growth inhibition may be caused by high osmolarity resulting from the feeding of highly concentrated yeastolate.

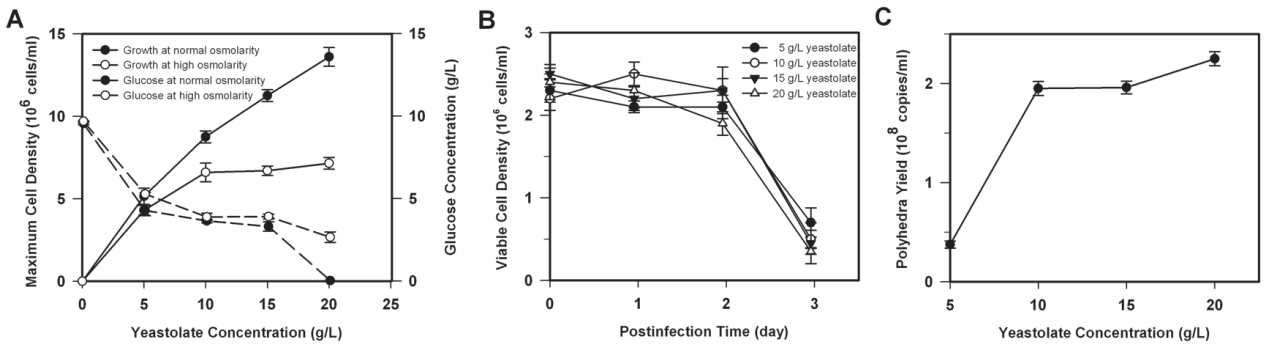

Figure 1: Effects of yeastolate on insect cell growth and polyhedra yield. (A) The maximum cell densities and residual glucose concentrations during Sf-21 cell growth in IBL-10 media with varied levels of yeastolate at either 340-350 mOsm/kg (normal) or higher osmolarity of 390-400 mOsm/kg. (B) The profiles of viable cell density for cell cultures infected in IBL-10 media with varied levels of yeastolate at a MOI of 1.0 ($TCID_{50}$/cell) for 3 days. (C) Effect of yeastolate on polyhedra yield after 3 days postinfection at an infection density of 2×10^6 cells/ml with a MOI of 1.0 ($TCID_{50}$/cell).

Effect of yeastolate on polyhedra production

In the following, all cell cultures in normal IBL-10 medium were grown to a cell density (2×10^6 cells/ml) and thereafter replaced with fresh culture media having elevated initial concentrations of yeastolate from 5 to 20 g/L during infection at a MOI of 1.0 ($TCID_{50}$/cell) to avoid nutrient depletion and ensure synchronous infection. Figure 1B depicts a two-phase infection process observed in the aforementioned cell cultures during postinfection, in which the cell densities in all the infected cultures stayed constant in the first two days of infection, where the cells stopped growth and their metabolism was completely regulated by virus replication and polyhedral production (O'Reilly et al., 1992). Thereafter, the cells were in the process of lysis and the viable cell count gradually dropped toward zero. A five-fold increase in polyhedra yield at 3 days postinfection was observed while yeastolate concentration was increased from 5 to 10 g/L in the medium (Figure 1C). Since ample and similar amounts of glucose and amino acids remained in these cultures (data not shown), yeastolate could possibly be the component limiting baculovirus replication and polyhedra production. Thereafter, further increase in yeastolate concentration did not result in significantly higher product yields. Thus, the concentration of yeastolate was maintained at 10 g/L in the culture medium for all the subsequent experiments.

Determination of molecular weight distributions of yeastolate and culture media

It is unclear which ingredients in yeastolate were responsible for the cell growth and polyhedra production, and whether these enhancing effects of yeastolate during growth and infection were due to the same ingredients. To tackle this issue, gel filtration chromatography was used to determine the molecular weight distributions of yeastolate and fresh/spent culture media during Sf-21 cell growth (Figure 2A) and baculovirus infection with fresh medium replacement (Figure 2B). Fresh and spent IBL-10 media and yeastolate are composed of substances with molecular weight below 12.4 kD. By comparing these molecular weight profiles further, we gained information on how yeastolate and the culture medium were utilized by Sf-21 cells during growth and infection. The cells seem to consume < 1 kD nutrients for growth (shaded area in Figure 2A). On the other hand, the infected cells utilized < 3 kD nutrients for cell maintenance and baculovirus replication (shaded area in Figure 2B), but most of nutrient consumption was still due to the uptake of < 1 kD substances. In view of the aforementioned differences in nutrient composition, the substances between 1 and 3 kD were likely the essential ingredients for baculovirus replication.

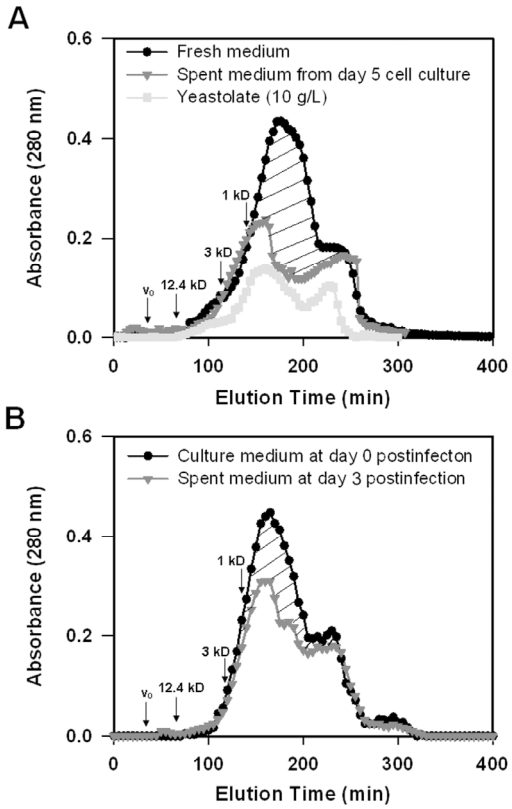

Figure 2: Comparison of molecular weight distributions of yeastolate and culture media. (A) Molecular weight profiles of yeastolate (10 g/L), fresh and 5-day spent culture media during Sf-21 cell growth. (B) Molecular weight profiles of fresh and 3-day spent culture media after baculovirus infection at 2×10^6 cells/ml with a MOI of 1.0. v_o denotes void volume of the column. The shaded areas under molecular weight distributions represent the differences in nutrient composition between fresh and spent media during cell growth and infection.

Fractionation of yeastolate

Based on the aforementioned gel filtration results, five molecular weight ranges of yeastolate fractions by membrane ultrafiltration and dialysis were used to evaluate which fractions could support significant cell growth and polyhedra production. Figure 3 shows that the components greater than 10 kD in yeastolate did not affect the cell growth and productivity. The cell growth was mainly

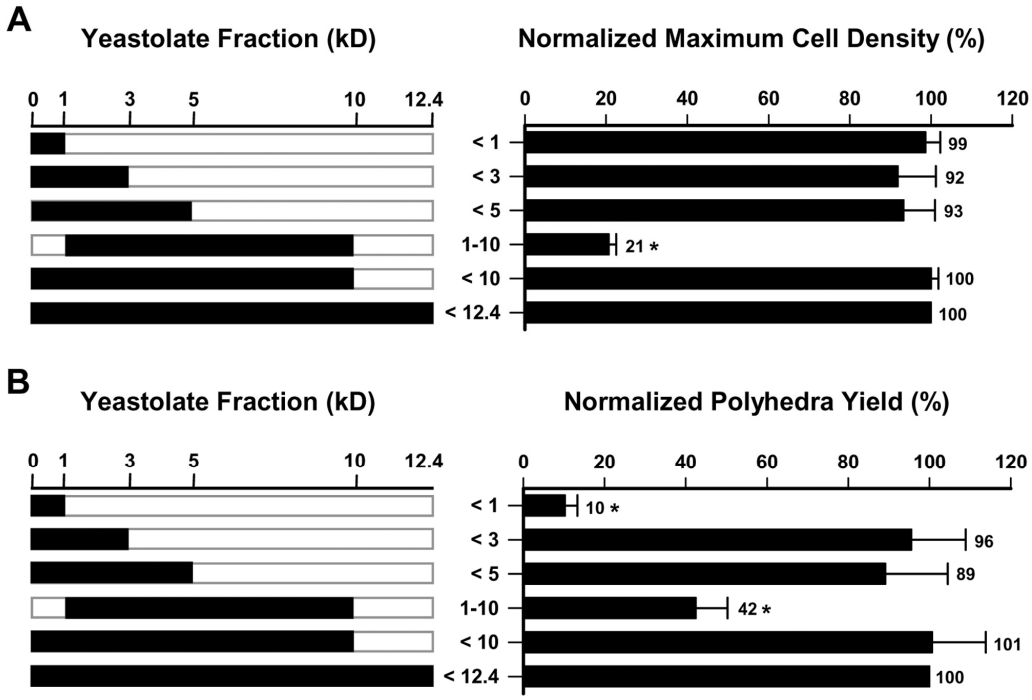

Figure 3: Identification of yeastolate fractions that support cell growth and polyhedra production. Yeastolate (10 g/L) was fractionated into five different molecular weight ranges of fractions to evaluate the effects of each fraction on (A) Sf-21 cell growth in terms of maximum cell density and (B) polyhedra yield at 3 days postinfection. For infection experiments, the cells were infected at 2×10^6 cells/ml with a MOI of 1.0 with medium replacement. The molecular weight range of each yeastolate fraction is denoted by the position of horizontal black bar along the axis of yeastolate molecular weight range. The numbers along the horizontal bars in the x axis represent normalized growth and productivity activities of the fractions compared to yeastolate (i.e. < 12.4 kD). *Significantly different, $p < 0.05$.

attributed to the uptake of < 1 kD yeastolate fraction, but the 1-10 kD faction also slightly supported the cell growth to only 21% growth capability compared to yeastolate (Figure 3A). This may be due to the entrapment of some residual yeastolate fraction below 1 kD during dialysis process. However, the < 1 kD fraction caused extremely poor polyhedra formation (10% compared to yeastolate, Figure 3B). Likely, certain components were needed for baculovirus replication and polyhedra production, and they may not exist in < 1 kD yeastolate fraction. Indeed, the components responsible for baculovirus replication can be traced to 1-3 kD yeastolate fraction (Figure 3B). Nevertheless, the infected cells still have to utilize < 1 kD yeastolate fraction for cell maintenance. Thus, the deletion of < 1 kD fraction from yeastolate (e.g., 1-10 kD yeastolate fraction) during postinfection resulted in decreased polyhedra yield (42% compared to yeastolate). The results clearly indicate that the cells utilize different nutrients for growth and baculovirus replication.

It has reported that synthetic peptides containing three to six amino acid residues or large oligopeptides can promote cell growth and product yields in animal cell cultures (Burteau et al. 2003; Franek and Katinger 2002; Franek and Fussenegger 2005). Since glucose and amino acids remained ample in Sf-21 cell cultures during growth and infection and the addition of other possible ingredients in yeastolate such as vitamins and nucleic acids did not successfully promote insect cell growth and product yield (Reuveny et al. 1993; Shen et al. 2007; Wu and Lee 1998), the enhancing effects of cell growth by < 1 kD fraction and productivity by 1-3 kD fraction might be likely attributed to larger molecules such as oligopeptides. However, further experimental exploration such as mass spectrometer analysis is needed for fully validating this possibility.

Conclusion

In the present study, Sf-21 cells were found to be incapable of surviving in yeastolate-free media. Elevated levels of yeastolate in media led to the increase in maximum cell density and polyhedra productivity. However, the feeding of highly concentrated yeastolate resulted in the reduced cell growth and polyhedra yield due to high medium osmolarity. Using membrane ultrafiltration and dialysis tubing separation techniques, bioactive ingredients in yeastolate were traced down to < 1 kD fraction for Sf-21 insect cell growth and 1-3 kD fraction for polyhedra production. The current finding provides a significant clue to further identification of specific bioactive elements in yeastolate for the development of fully chemically-defined serum-free media.

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