Development of an Efficient Bioprocess for Poultry Vaccines Using High-density Insect Cell Culture[a]

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INTRODUCTION

Infectious bursal disease virus (IBDV) is a pathogen of major economic importance to the world's poultry industries. It causes severe immunodeficiency in young chickens by destroying the precursors of antibody-producing B cells in the bursa of Fabricius.[1] IBDV is a member of the Birnaviridae family whose genome consists of two segments of double-stranded RNA. Presently, the principle method of controlling IBDV infection in young chickens is by vaccination with an avirulent strain of IBDV or by the transfer of high levels of maternal antibody to breeder hens.[2] Recently, virulent strains that are antigentically different from previously established IBDVs have been isolated from vaccinated flocks on the Delmarva peninsula. Consequently, present vaccines afford only partial protection against infection.

In FIGURE 1, the large segment indicated is comprised of a precursor polyprotein that is processed into mature VP2, VP3, and VP4.[3] VP2 and VP3 are the major structural proteins of the virion. VP2 is the major host-protective immunogen of IBDV and contains the antigenic regions responsible for the induction of neutralizing antibodies. For this reason, we have focused on VP2 as a potential vaccine. When expressed in baculovirus as one of a cassette (VP2, VP3, and VP4), the coat proteins self-assemble into empty virions, which subsequently afford protection in challenged chickens (FIG. 2).[4]

This article presents the current status of our efforts to develop a cost-efficient vaccine for IBDV using the recombinant baculovirus and insect cell culture process. Although the baculovirus expression system has proven quite successful for use in laboratories, its use as an industrial expression system has yet to occur, although products for human use are in clinical trials. Briefly, the system entails replacing the gene for the AcNPV coat protein, polyhedrin, with the gene for the protein of

[a]This research was supported in part by Grants from the U.S. Department of Agriculture (no. 90-34116-5399), and the Maryland Agricultural Experiment Station (scientific article no. A-6474, contribution no. 8679). M-Y. Wang was supported by a Center for Agricultural Biotechnology Fellowship Award.

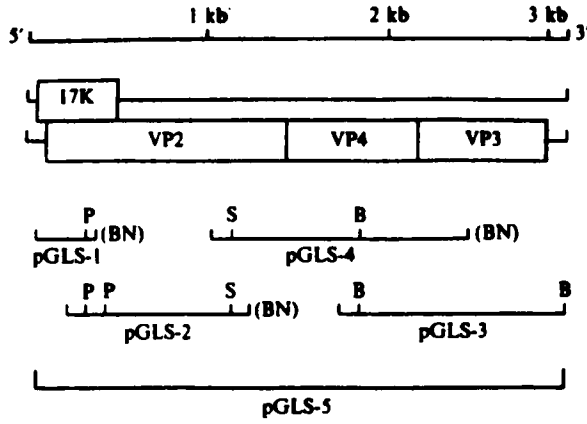

FIGURE 1. Schematic of IBDV genome and baculovirus constructs. A preprotein consisting of VP2, VP3, and VP4 is processed into VP2 and VP3 structural proteins. The vaccine is based on the VP2 coat protein.[4]

interest. This coat protein is not required for the replication and maintenance of the virus in cell culture due to its biphasic life cycle. About three days after infection with a recombinant virus, the protein of interest is expressed instead of polyhedrin, which would normally constitute the majority of the total cellular protein. The system is attractive because it is nonpathogenic towards humans, and it involves the *in vitro* propagation of cells for which technology has advanced due to previous mammalian and hybridoma culturing techniques. It provides for an abundant supply of the protein of interest in part due to the strong polyhedrin promoter and also because insect cell lines reported to date provide for glycosylation and other posttranslational processing. In addition, the construction of recombinant baculoviruses has become simplified due to commercial expression kits. Several general articles, manuals, and review articles have been published that describe both the expression system[5–8] and novel process engineering aspects.[9–11] In this work, we subdivide the entire bioprocess into three key areas: (1) metabolism of infected cells and specific heterologous protein yield; (2) bioreactor configuration and high cell density continuous culture; and (3) integration of expression and product separation. In each of the RESULTS subsections, we briefly review the recent literature as well as present our efforts to understand and advance these areas.

MATERIAL AND METHODS

Cells, Media, and Culture Conditions

Unless otherwise noted, Sf-9 cell stocks were cultured with Ex-cell 401 medium in spinner flasks (Bellco, No. 1967-00250 (170 × 85 mm, 250 mL) and were transferred from T-flasks after viable cell count using Trypan blue. Gentamycin and

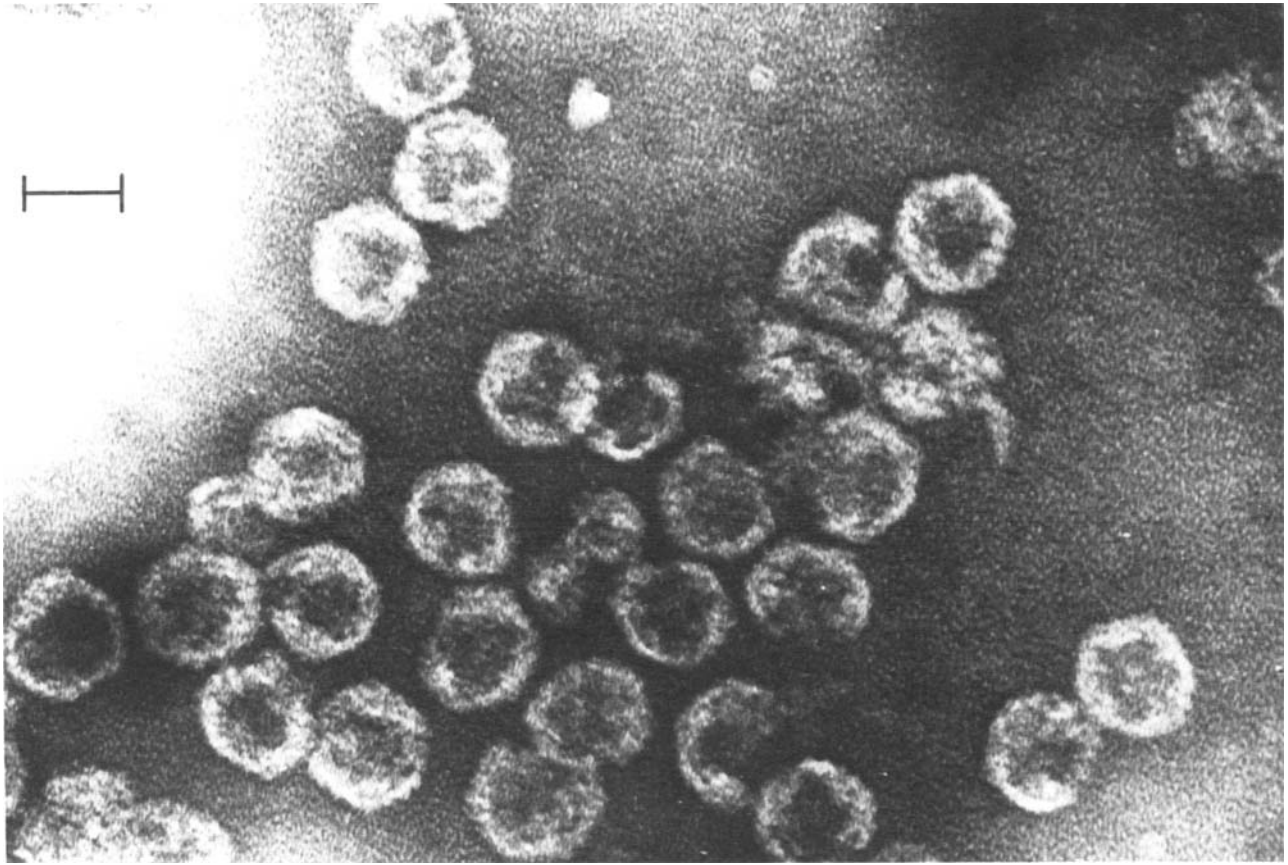

FIGURE 2. Electron microscopy of purified IBDV VLPs (260,000×) from Sf-9 cells infected with a recombinant baculovirus, vIBD-7. Bar length denotes 50 nm.

amphotericin (2.5 mg/mL) were not added to suspension cultures. A 3-L Applikon fermentor with I-L working volume was equipped with a pitched-blade low-shear impeller and automatic pH dissolved oxygen (DO) control through regulation of the headspace nitrogen, oxygen, carbon dioxide, and air gas composition. This controller was assembled in-house. The oxygen flow rate was manually adjusted to assist in maintaining DO at the required percent of air saturation, and the agitation rate was routinely 65 rpm. Inoculum was prepared from 250 mL spinner flasks. The pH was controlled to above 6.3 by carbon dioxide. Base was not used to maintain pH, thus there was no change in osmolarity. For continuous culture, a glassblown-jacketed spinner flask was constructed and was quite successful as a low-volume cell-growth bioreactor. Temperature was maintained at 28°C using a circulating water bath (VWR). The top was covered by a rubber stopper (No. 10) drilled with seven holes for stirring, media inlet and outlet, gas flow, sampling, level control, and vent. The agitation system was comprised of a glass rod and stir bar, driven by a magnetic stirrer (Bellco, NJ). The sparging system consisted of a porous HPLC solvent sparger (Waters) connected to No. 13 silicon tubing (Masterflex). Air was introduced with a peristaltic pump (Cole Parmer) in an on/off cycling mode using a 14-hr home timer device.

Viruses and Viral Infections

The stock virus solutions were developed as described previously.[4] Virus titer was determined by the end-point dilution method. The infections were performed by adding different volumes of virus solution after viable cell count. Thus, the time postinfection commences from the addition of the virus solution. The multiplicity of infection (MOI) was evaluated separately in spinner flask culture. Samples (2 mL) were taken from fermentors during the infection process, divided in two tubes, and centrifuged for 20 min at 12,000 rpm. The supernatants were separated from cell pellets and stored at −20°C until measurement of glucose and lactate. The cell pellet was resuspended in 250 mL 10 mM KH_2PO_4 (pH 7.4) buffer solution and stored at −20°C until assayed (PAGE) for quantification. Another two 10 mL samples were centrifuged for 20 min at 3500 × *g*. The cell pellets were stored at −20°C without resuspending in any buffer and used for the measurement of proteolytic activity and total protein.

Analysis

Total cell counts were performed with a VWR-brand hemacytometer, and viability was determined by Trypan blue dye exclusion using a 0.04% solution (Sigma). Cell pellets resuspended in 250 mL EHBS were sonicated on ice for 10 s with a microtip and a 30% pulsed duty cycle. Glucose and lactate were measured using a YSI Model 27 analyzer (Yellow Springs Instruments), and ammonia was determined by an enzyme-based assay kit (Sigma, No 170-UV). Total protein was measured using a BioRad protein assay kit I. Alkaline protease activity was assayed using the synthetic substrate azocasein (Sigma) at a concentration of 2% (w/v) in 0.1 M Tris/HCl buffer (pH 9.0). The cell pellets were resuspended using 250 mL of 0.1M

Tris/HCl buffer (pH 9.0) and sonicated. One milliliter of azocasein solution was added to this 250 mL sample, and this mixture was incubated at 28°C for 1.5 hours. The reaction was stopped by the addition of 10% trichloroacetic acid (1.2 mL). After standing at 40°C for 30 min, the mixture was filtered, and 0.5 mL 0.5 M NaOH was added to 0.5 mL of the filtrate. One unit of alkaline protease activity is defined as the amount of enzyme that gives an increase in A_{440} of 0.1 in 30 min at 28°C.

Immobilized Metal Affinity Chromatography (IMAC) Separation

Cells were harvested by centrifuging 70 mL of culture and then resuspending them in 10 mL native binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8). Egg white lysozyme (100 μg/mL) was added to this mixture, which was incubated for 15 minutes on ice. The cells were sonicated on ice three times for 10 s with a 30% pulsed duty cycle (Branson Sonic Power, Co.) and then flash frozen in a −80°C freezer. After thawing the sonicated cells at 37°C, a final concentration of 5 μg/mL, RNase was added to the crude cell lysate and incubated on ice for 15 minutes. Insoluble cell debris was removed by centrifugation at 3,000 *g* for 15 minutes. The column resin (Invitrogen, CA) was washed with distilled water and then equilibrated with native binding buffer. Cell lysate (7 mL) was then loaded onto the column and washed by native binding buffer until OD_{280} was less than 0.01. A step down to pH 6.3 was performed using native wash buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 6.3), and the OD_{280} of supernatant was monitored until its value was smaller than 0.01. Protein was then eluted by loading native-pH elution buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 4.0) onto the column and collecting 1 mL fractions.

RESULTS

Metabolism of Infected Cells

Hundreds of laboratories have expressed heterologous proteins using recombinant AcNPV and Sf-9 or Bm-5 cell lines, yet only several laboratories have focused attention on maximizing yield by examining cellular functions during the growth and expression phases. Factors such as MOI, timing of infection, and cell density have been well documented. For example, Neutra *et al.*[12] examined the effects of reactor volume, infection timing, MOI, and cell density on β-galactosidase production in shake flasks. King *et al.*[13] found that low MOI was favorable for a temperature-sensitive baculovirus. Conversely, for a more typical baculovirus with polyhedrin promoter control and an rCD4 product, Lazarte *et al.*[14] found high MOI was most favorable. Perhaps these systems differed by changes in cell metabolism that resulted from medium condition and infection temperature. In the Lazarte *et al.*[14] study, fresh medium added at infection tripled the yield. Indeed, several groups have demonstrated that infected cells retain significant metabolic activity, and this is essential for producing the recombinant protein. Caron *et al.*[15] noted that postinfection cell physiology was critical and suggested that different growth and production medias might be required. Zhang *et al.*[16] noted that for Bm-5 cells, serum concentrations for

optimal growth were different than for viral production. Additionally, Kamen *et al.*[17]quantified differences in principal nutrients and waste products during the growth and production phases.

Reports demonstrating the effects of nutrient limitation and by-product inhibition on protein yield have been limited, however. Wang *et al.*[18] demonstrated that apparent glucose limitations could be offset by subsequent sucrose decomposition. Lindsay and Betenbaugh[19] noted that the oxygen demand was higher for infected cells and adjusted aeration properties, which facilitated dramatic increases in yield. Likewise, Scott *et al.*[20] reported a significant increase in protein yield by maintaining dissolved oxygen tension at 50% of saturation. However, at high cell densities, maintaining oxygen was not sufficient for increasing yield. Caron *et al.*[15] restored recombinant protein production at higher cell density by renewing the medium at the time of infection. Feeding the specific rate-limiting nutrients is less expensive, however, and aides in elucidating insect cell physiology under viral infection.

Oxygen

Two batch cultures were conducted to examine the effects of DO during viral replication and protein production.[21] The first batch culture was in a fermentor without DO control (FIG. 3), while the other was conducted with DO regulated near 35% (FIG. 4). Virus infection was performed for the fermentor without DO control by adding 15 mL virus solution (MOI 5) at 91 hours, after the cells had attained a concentration of 6.15×10^5 cells/mL. This MOI and cell density were chosen so that other factors, such as nutrient depletion, were eliminated and differences due to oxygen level were discernible. Shortly after infection, the DO dropped to zero and remained zero for another 90 hours. The viable cell concentration remained near 6.55×10^5 cells/mL and then started to decrease simultaneously with an increase in DO after 190 hours (FIG. 3a). The onset of cell lysis, however, is marked by both the increment in lactate dehydrogenase (LDH) activity and decrement in cell viability near 150 hours (FIG. 3b). Note that after the DO dropped to zero, the lactate increased to a maximum of 2.5 mM, reflecting the inadequacy of oxidative phosphorylation to supply the required ATP. In these energy-limited cells, the final EH activity was 0.015 units per milliliter.

For the batch culture with regulated DO, the growth kinetics are shown in FIGURE 4. Virus infection was also performed at 90 hours and 6.55×10^5 cells/mL. Unlike the oxygen-uncontrolled culture, the viable cell concentration continued to increase to a maximum of 1.05×10^6 cells/mL. The increased demand in oxygen immediately following infection was met in this experiment, and the DO was maintained above 10% and averaged 35% for the remainder of the experiment. The continuous addition of oxygen likely provided the requirement for continued cell growth. Note that the lactate concentration remained below 1 mM for the entire experiment, whereas the glucose concentration dropped almost 45%, from 14 mM to 8 mM after infection. The epoxide hydrolase activity reached 0.044 U/mL, which was 200% higher than in the oxygen-uncontrolled experiment. The specific productivity increased by 100% as well. This increase in protein synthesis is accomplished only by continued glucose uptake after infection. In the uncontrolled experiment, infected cells consumed little glucose (~ 1 mM) after the DO dropped to

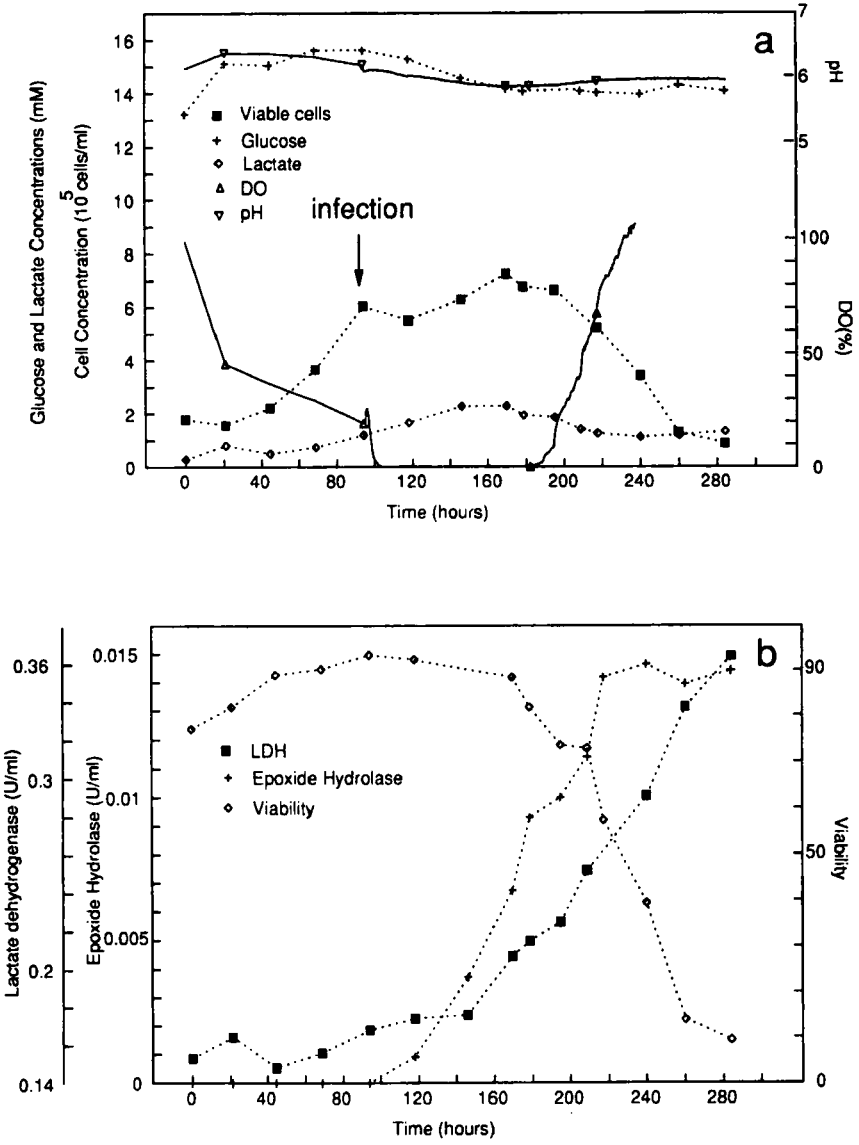

FIGURE 3. Batch culture of SF-9 cells grown without DO control. **a:** Glucose, DO, lactate, and viable cell concentrations and pH are plotted versus time. At time indicated by arrow, cells were infected (MOI 5). **b:** Epoxide hydrolase (EH), lactate dehydrogenase (LDH), and viability are depicted.[21]

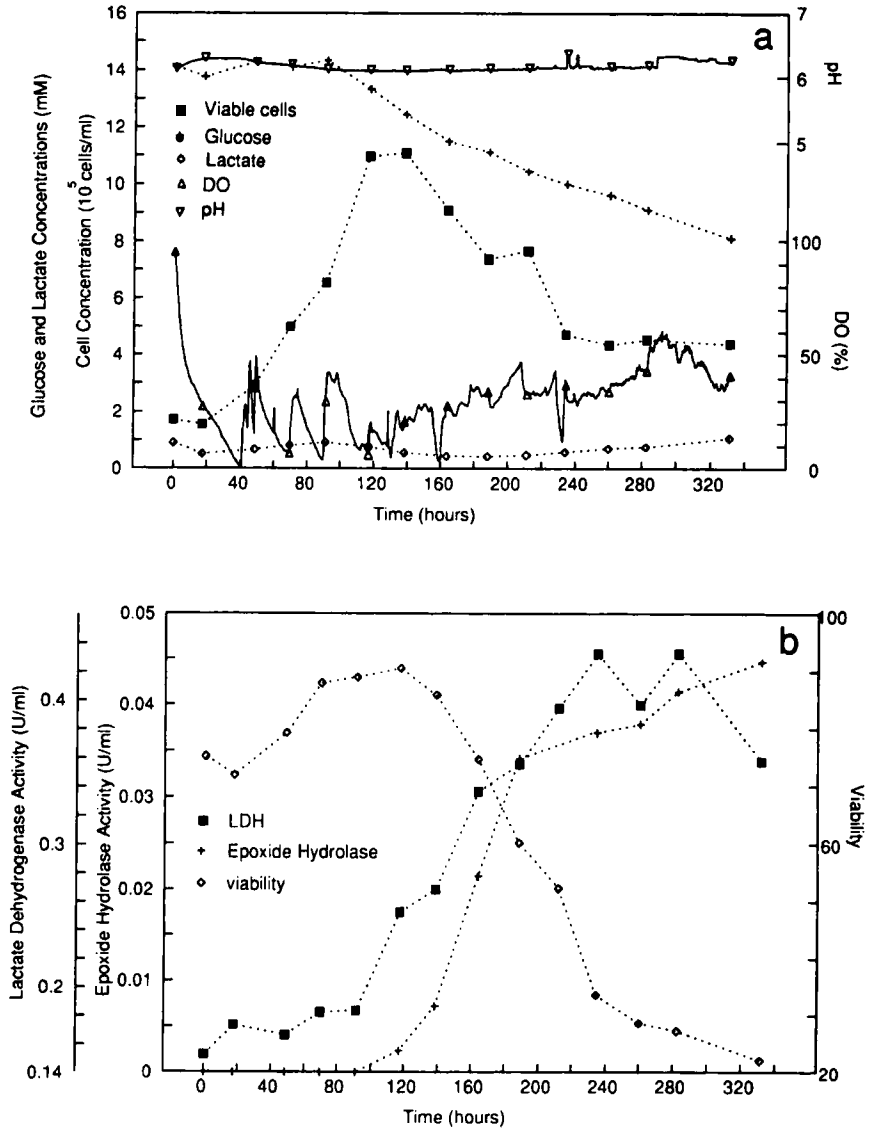

FIGURE 4. Batch culture of Sf-9 cells grown with regulated DO (average 35%).[21] **a:** Glucose, lactate, DO, and viable cell concentrations and pH are indicated. **b:** EH, LDH, and cell viability are included, as noted in legend.

zero. On the other hand, both the uninfected and infected cells in the DO-controlled experiment continued to uptake glucose and consumed almost 1 g/L after infection for cell growth and viral replication. Note, therefore, that the effects of oxygen, like MOI and cell density, are delineated and realized when sufficient nutrients are available in the extracellular environment.

Glucose/Glutamine

Three experiments were performed to evaluate glucose and glutamine feeding in spinner flasks at high cell density (FIG. 5). The use of 50 mL spinner flasks with 30 mL working volume prevents oxygen limitation. The cells were grown in a 250 mL spinner flask with 80 mL working volume until a density of 3.85×10^6 cells/mL was attained; they were then divided and infected in three smaller spinner flasks at an MOI of 10 and a cell density of 3.3×10^6 cells/mL. One of the spinner flasks was diluted 3:1 with fresh medium as a control (total working volume, 30 mL). Previously, this method was reported as resulting in the highest specific yield attainable in spinner flasks.[15,19] This spinner flask is denoted an oxygen and substrate-sufficient control. One spinner flask was then fed 0.5 mL of 200 mM glutamine and 80 μL of 2 M glucose. Subsequent daily glucose feeding maintained glucose supply (FIG. 5a). The last spinner flask was a control high cell density culture, without glucose/glutamine feeding. The epoxide hydrolase activity (FIG. 5b) continued to increase for the culture with fresh medium (oxygen/substrate control) until 118 hours postinfection (hpi). By contrast, in the high cell density cultures, epoxide hydrolase activity increased more rapidly initially and then decreased at 88 hpi for the glucose/glutamine fed culture, and at 48 hpi for the unfed culture. A significant improvement in protein production from glucose and glutamine feeding was seen as early as 12 hpi. Coincidentally, the glucose concentration in the unfed culture had dropped dramatically by this time (FIG. 5a). The increase in activity continued well into the production phase and because the viable cell concentration in the fed culture was significantly lower after 48 hpi, the increase in activity was due primarily to an increase in specific productivity.

When comparing the overall performance of the effects of oxygen, glucose, and glutamine feeding, the total protein activity of the DO-controlled culture was three times that of the uncontrolled culture. Further, the glucose/glutamine fed culture was twofold higher in yield than the DO-controlled culture. Ultimately, the epoxide hydrolase yield was 40–60 milligrams per liter.[22]

Protease

As seen above, cell metabolism may play a very important role in recombinant protein production when cells are infected at high cell density. The metabolic stress responses of bacterial and mammalian cells to oxidation, heat shock, and amino acid starvation have been well documented, and many stress proteins have been identified.[23,24] Insect stress, however, has been quantified by previous researchers only by measuring the bulk proteolytic activity of whole insect homogenates.[25,26] In those reports, increased proteolytic activity corresponded to increased cellular stress.

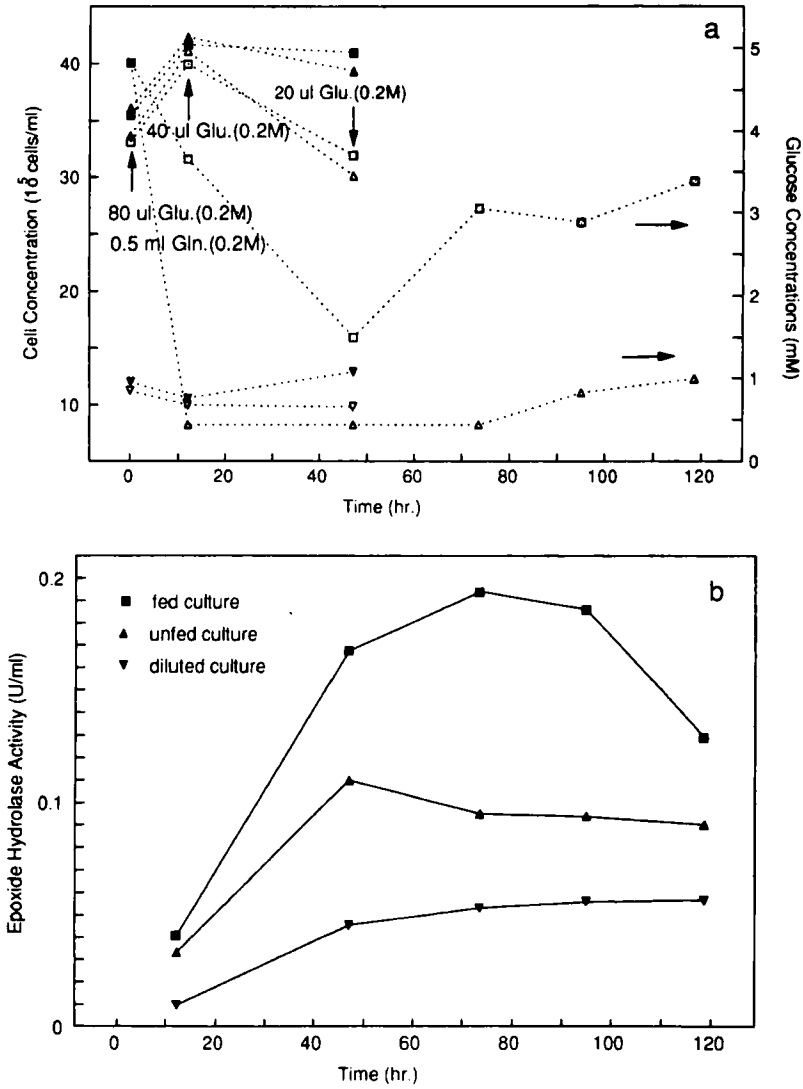

FIGURE 5. Experiment depicting glucose and glutamine feeding in spinner flasks.[21] **a:** Cell density and glucose concentrations; feeding indicated by arrows. Unfed high-density culture: viable cells (△), total cells (▲), glucose (◬). Fed high-density culture: viable cells (□), total cells (■), glucose (▨). Low-density diluted culture: viable cells (▽), total cells (▼). **b:** EH activity in same spinner flasks.

In many recent reports, degradation of the recombinant product has been significant.[20,27,28] Thus, another important aspect of cell metabolism concerns the elicitation of protease activity. We have used azocasein to investigate the appearance of alkaline proteases that may breakdown the product, particularly if allowed to follow in the separation and purification processes.[29]

In another fermentor experiment, we controlled the dissolved oxygen to 50% and infected cells (MOI 5) by the addition of virus stock solution when the cell density was about 1.2×10^6 cells/mL. This lower cell density was chosen in order to avoid other cellular stress effects (for example, nutrient starvation). The cells (FIG. 6) kept growing after viral infection due to a low MOI[21,30] for a period of 40 hours and then the viable cell density decreased later due to a secondary infection. The total cell density decreased due to cell lysis, and the viability was 20% at cell harvest. Two recombinant protein activities and the total specific protease activity are also shown. In this system, β-galactosidase expression is under promoter P10 control; epoxide hydrolase (EH) is our desired recombinant product and is under the polyhedrin promoter control. β-galactosidase is a cytoplasmic protein and will be liberated to the medium during expression and lysis. However, EH is membrane bound and not released to the medium. Expression of both proteins (FIG. 6) started simultaneously. The β-galactosidase activity increased until 79 hpi, was constant for a short time, and then decreased by 12% until harvest. On the other hand, EH increased more slowly initially, remained constant for 20 hours and increased slowly by 20% until harvest. The specific protease activity was shown to increase dramatically starting at about 70 hpi. This time just corresponds to the reduction of β-galactosidase activity and the slowdown in EH production. We should note that the viability was near 70% at this moment. Inasmuch as the viability was high and there was no nutrient limitation (not shown), there was no apparent reason for cells to stop producing recombinant protein. The protease (measured as specific activity in the cell pellet) increased at least 15-fold even when considering partial loss from cell lysis. We also measured proteolytic activity in supernatant, and no increment was found, which suggests that the proteolytic activity was either associated with the cell membrane or was too dilute to detect in the supernatant. Although there is no specific connection between the protease activity and the shift in protein activities (β-gal, EH), the coincident timing warrants further consideration. Because it is unlikely that protease inhibitors will be used in a commercial process, the specific nature and timing of this proteolytic activity is significant. Hence, characterization of the identity and consequences of this and other proteases is under investigation.

Bioreactor Configuration and Continuous Culture

In addition to the many productivity advantages that continuous suspension culture has over stationary (T-flask) or batch-suspension culture, continuous culture provides homogeneous and constant environmental conditions that facilitate the modeling of cell growth and metabolism. Research with hybridoma cells has demonstrated this point.[31,32] Due to infected cell lysis, most continuous insect cell systems have employed two reactor stages: one for cell growth and the other for virus propagation and recombinant protein expression.[11,33–38] However, it has been re-

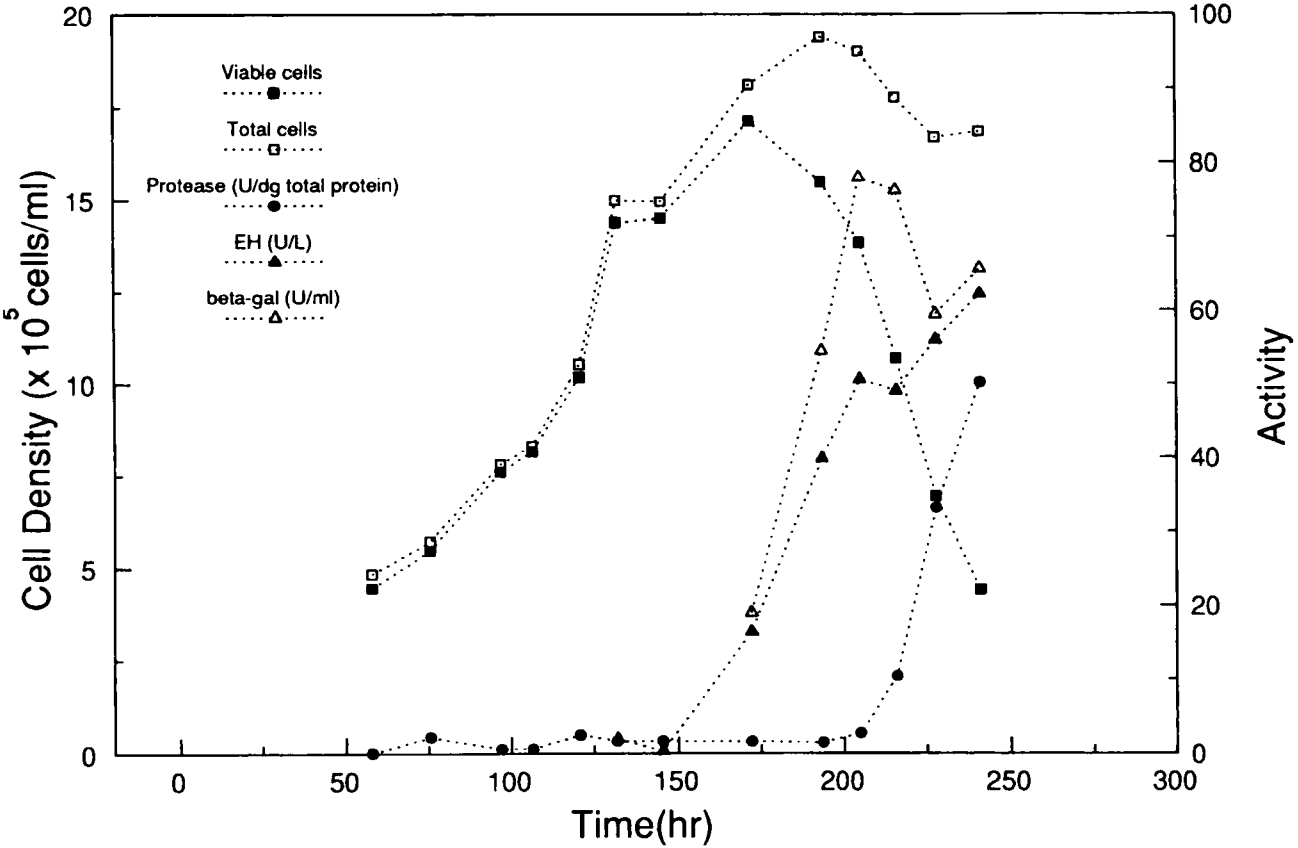

FIGURE 6. Analysis of alkaline protease activity during postinfection period of fermenter, with DO controlled to 50% of air saturation. Viable and total cell concentrations, specific protease activity, and EH and β-galactosidase activity are depicted.[29]

ported that there is eventually an almost complete loss of productivity from continuous insect cell culture with a continuously passaged virus.[34,35,38] Limited stability of the virus upon serial passage may limit the application of two-stage continuous reaction systems. This may be improved, however, by a three-stage continuous bioreactor system proposed by Taticek and Shuler,[38] or by a cycling-batch reactor scheme.[11] At present, batch and perfusion systems have achieved significantly higher viable cell density than continuous cultures. Further, there is some question as to the long-term productivity of the cell lines in continuous cultures, independent of the virus.[15] Consequently, we have investigated methods for maximizing cell number in extended continuous cultures.[39] Dilution rates were examined from 0.014 to 0.0403 hr^{-1}, which is close to the maximum specific growth rate obtained in batch cultures.[17]

In FIGURE 7, our small-volume glass-jacketed spinner flask reactor[39] is depicted. Cultures were stirred at 130 rpm and contained 0.1% pluronic F-68.[40] The air-sparging system was cyclic with 60 min on and 30 min off. Two volummetric air flow rates were examined: 2.056 mL/min (0.0093 vvm) and 2.76 mL/min (0.0125 vvm) for low- and high-sparging rates, respectively.

The viable and total cell densities for both sparging rates are plotted as a function of dilution rate in FIGURE 8. At the low sparging rate, a monotonic decrease in viable cell density with increasing dilution rate was observed. This is consistent with the semicontinuous culture of mouse LS cells (without pH and DO control) by Sinclair.[41] The high sparging rate exhibited a maximum in total and viable cell densities at a dilution rate of 0.0287 hr^{-1}. The decrease in viable cell density at lower dilution rate (0.0251 hr^{-1}) may have been due to low nutrient and/or high toxin concentrations.[31]Note that at 0.0332 hr^{-1}, the cell density for the high sparging rate (0.0125 vvm) was five times higher (3.64×10^6 vs. 7×10^5 cells/mL) than the low sparging rate. Thus, this sparging rate provided much better oxygenation for cell growth with little contribution to cell death. The trends shown in FIGURE 8 are also similar to a continuous sp2/0-derived mouse hybridoma cell culture with pH and DO control[31] as well as an oxygen-limited continuous culture (pH control only) of mouse NBI hybridomas.[42] The data depicted was collected in one continuous culture of over 3100 hours in duration. At several times, the dilution rate was reset to a previous setting, and the cell density returned to the previous steady state value.[39] Thus, the spinner flask reactor performed well, was reproducible, and will be very useful for further development of the additional protein expression stages.

Recombinant Protein Expression

In FIGURE 8, we demonstrated that a cell density of 4.2×10^6 cells/mL with 94% viability could be achieved at a dilution rate of 0.0287 hr^{-1}. In order to check the protein expression level of infected cells grown continuously for an extended period, cells at dilution rate of 0.0251 hr^{-1} with low (0.0093 vvm, 800 h) and high sparging rate (0.0125 vvm, 2600 hr) were taken from the bioreactor and directly infected with an MOI of 5 in two 50 mL spinner flasks (30 mL working volume). The maximum recombinant protein yields were 0.15 U/mL at 71 hpi and 0.05 U/mL at 69 hpi for cells from the 2600 hr and 800 hr samples, respectively. The maximum cell densities reached were 3.7×10^6 and 1.6×10^6 cells/mL for the 2600 hr and 800 hr samples.

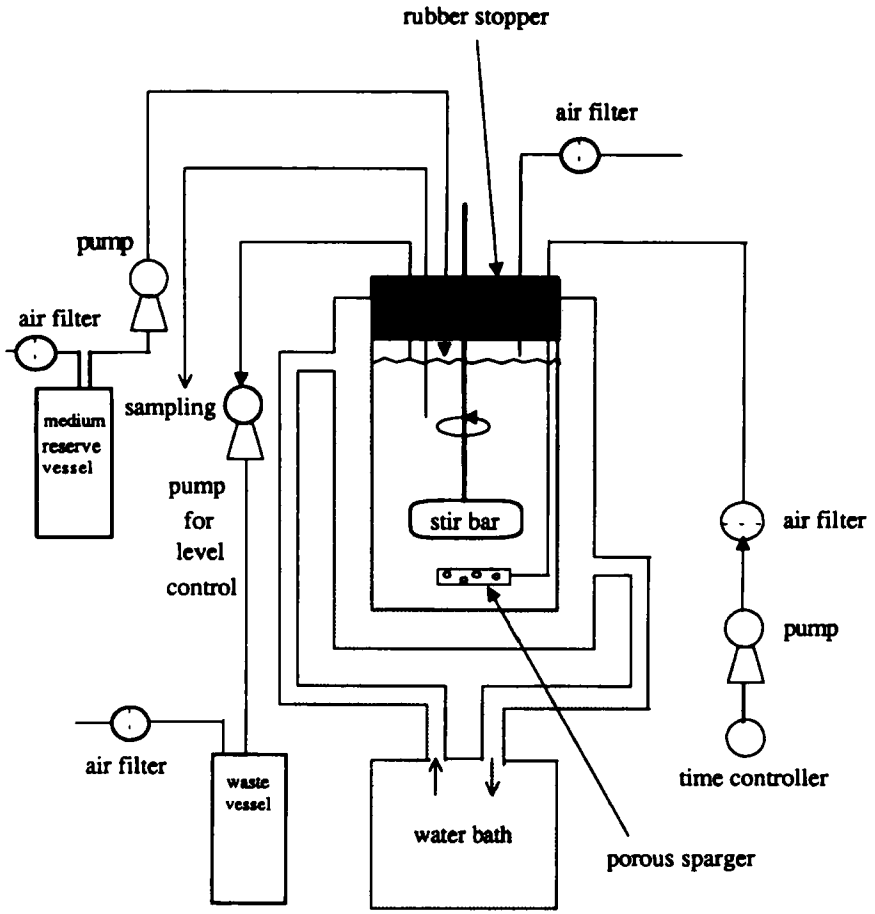

FIGURE 7. Flow diagram and setup of continuous bioreactor system. The total volume is 250 mL, with working volume up to 230 mL. Air is introduced by way of peristaltic pump and HPLC solvent porous sparger. Air is introduced in on/off mode with a home timer. Temperature is maintained by water bath and glass jacket. Agitation is provided by magnetic stir plate (Bellco).[39]

The average specific EH activity, calculated as the maximum enzyme activity divided by the maximum cell density, was slightly lower for cells from the lower sparging rate (3.125 vs. 4.05 × 10^{-3} U/10^5 cells). Perhaps this was due to a lower growth rate (0.022 hr^{-1} vs. 0.025 hr^{-1}) or lower oxygen supply during the continuous culture. However, the specific EH activity from these long-term cultured cells is comparable to that from freshly prepared cells (3.34–5 × 10^{-3} U/10^5 cells[21]).

This demonstrates that high cell density (more than 3 × 10^6 cells/mL) can be achieved in continuous culture, even at a dilution rate of 0.0403 hr^{-1}. In addition, the

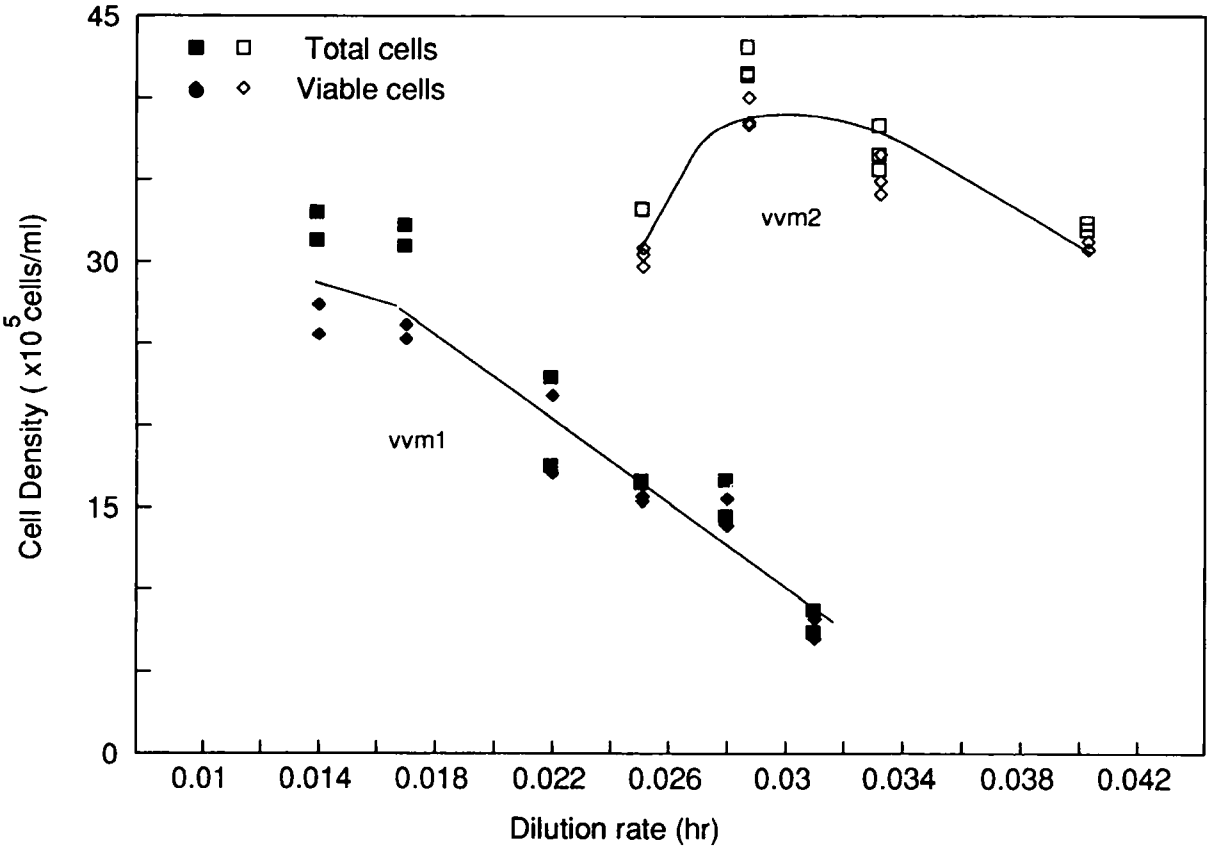

FIGURE 8. Steady-state viable (squares) and total (solid squares) cell concentration for continuous cultures at high and low sparging rates. Glucose and glutamine were not growth limiting (not shown).

specific protein yield from long-term cultured cells (over three months) is not compromised. The results of this work can be combined with the previous discussion on maximizing specific productivity in designing the best reactor strategy. This may involve incorporating feeding policies in the second or third stage of a multistage continuous system, or the inclusion of several alternating batch reactors that are filled by one continuous or semicontinuous reactor.

Integrated Expression and Separation for Vaccine Development

An attractive feature of the baculovirus system has been its potential for disease diagnostics and vaccine development. Again, this is due to the high expression level, posttranslational processing, and nonpathogenicity. In addition, recent reports have shown that expression of virus coat proteins often results in self-assembled virus-like particles (VLP) that are essentially empty whole virions.[4,43–51] Of these VLP-producing systems, vaccines have been proposed for poliovirus,[49] parvovirus,[50] IBDV,[4] flock house virus,[51] and bluetongue virus.[44] In addition, vaccines have been proposed or tested from non-VLP proteins, including HIV,[52] yellow fever,[53] feline herpes virus,[54] *Trypanosoma cruzi*,[55] Mokola and rabies viruses,[56] Newcastle disease virus,[57] Japanese encephalitis,[58] hepatitis C,[59] and bovine coronavirus.[60] Of these, only two research groups have considered the advantages of integrating initial separation with product expression by employing IMAC.[59,61]

We have developed the bioprocess so that IMAC can be employed as a potentially single-step separation procedure. IMAC was introduced for the selective adsorption of protein by Porath *et al.*[62] who used divalent cations, Zn^{2+} and Cu^{2+}, chelated to a chromatographic matrix to fractionate serum proteins. Metal ion ligands most often used in IMAC are first-row transition metals (Zn, Ni, Cu, and Fe) chelated by iminodiacetate (IDA). In resin, they distinguish affinities exhibited by functional groups on the surface of proteins. These interactions between particular surface amino acids and immobilized metal ions provide the basis for metal-affinity protein separations.[63–65] To a first approximation, proteins are retained on metal-affinity columns according to the number of accessible histidines.[66,67] Histidine is not a common residue in protein sequences and it accounts for only 2.1% of amino acids in globular proteins.[68] Thus, the chance that a desired protein, engineered to contain additional histidines at its C- or N-terminus, will be preferentially separated from other proteins is quite good.

A description of the construction of the VP2 baculovirus that expresses His_5-VP2 (denoted VP2H) is given elsewhere.[61] Monoclonal antibodies (mAbs) recognizing IBDV were produced and characterized using protocols previously described.[69,70] Identification of IBDV antigens by modified antigen-capture ELISA (AC-ELISA) was also carried out as described by Snyder *et al.*[71] Recombinant VP2 proteins from insect cells infected with vEDLH-22 (for VP2H) and vEDL-8 (for VP2) were identical using the array of mAbs and AC-ELISA. However, these two proteins (VP2, VP2H) were distinguished and resolved on a 12.5% SDS-polyacrylamide gel, and detected immunologically following Western blotting with polyvalent chicken anti-IBDV serum.[61,72] This indicated that VP2 protein was fused with histidine residues.

The VP2H was purified by affinity chromatography using a Ni^{2+} immobilized resin column provided in a protein purification kit as described in the METHODS section (Invitrogen, CA). The final elution was monitored by OD_{280} readings of the fractions, using the native-pH elution buffer as a blank. In FIGURE 9, the optical density (OD_{280}) of the column eluate is shown for the wash and VP2 eluate steps. The eluted pool (fractions 18–26) from the pH 4 buffer was collected, concentrated, and desalted by a 10 kDa membrane (Amicon) and checked by Western blotting.[61]

In FIGURE 10a, the purified VP2H protein (molecular weight near 43 kDa, lane 2) comigrated with two VP2 proteins: one derived from GLS IBDV (lane 1) and the other from Sf-9 cells infected by IBDV-7 recombinant baculovirus[4] encoding entire VP2, VP3, VP4, VP2X proteins of IBDV (lane 5). This demonstrates that the VP2H protein can bind the Ni^{2+} ion on the resin and was effectively eluted by the pH 4 elution buffer. The purity of purified VP2H protein was also checked by SDS-PAGE (FIG. 10b). The intense band with a molecular weight of about 43 kDa showed that this VP2H protein was dominant in the mixture (roughly 40%). Some small proteins or peptides were still in this purified mixture which may have been due to partial degradation of VP2H (data not shown) or additional elution by this strong low-pH buffer. In this work, a step change from pH 6.3 to pH 4, which is much lower than the pKa value commonly observed for surface histidines (~6.0),[73] may have coeluted some peptides with a stronger binding capacity.[74]

Because we demonstrated that the recombinant viruses express immunoreactive VP2 proteins, the next step was bench-scale production of VP2H protein. Cells were infected with an MOI of 3 when the cell density was approximately 2.7×10^6 viable

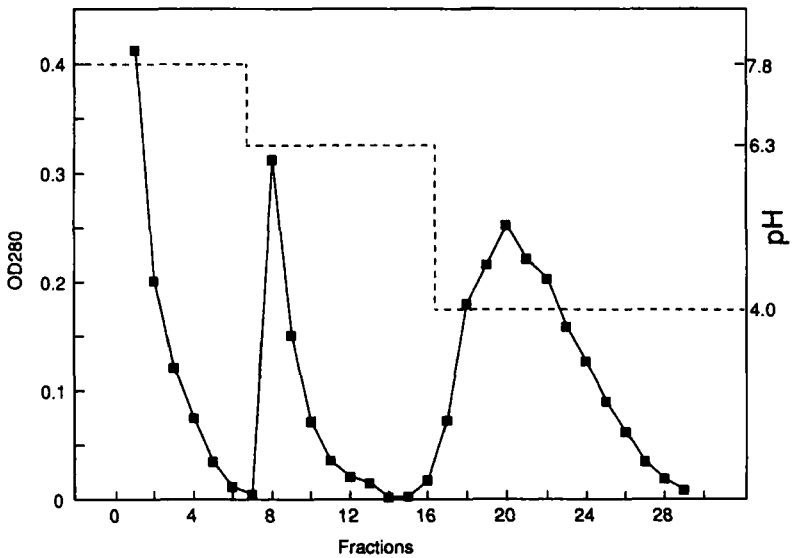

FIGURE 9. Optical density (280 nm) elution profile of Ni(II)-IMAC column. Fractions 18–26 were pooled together for gel electrophoresis and Western blotting.[61]

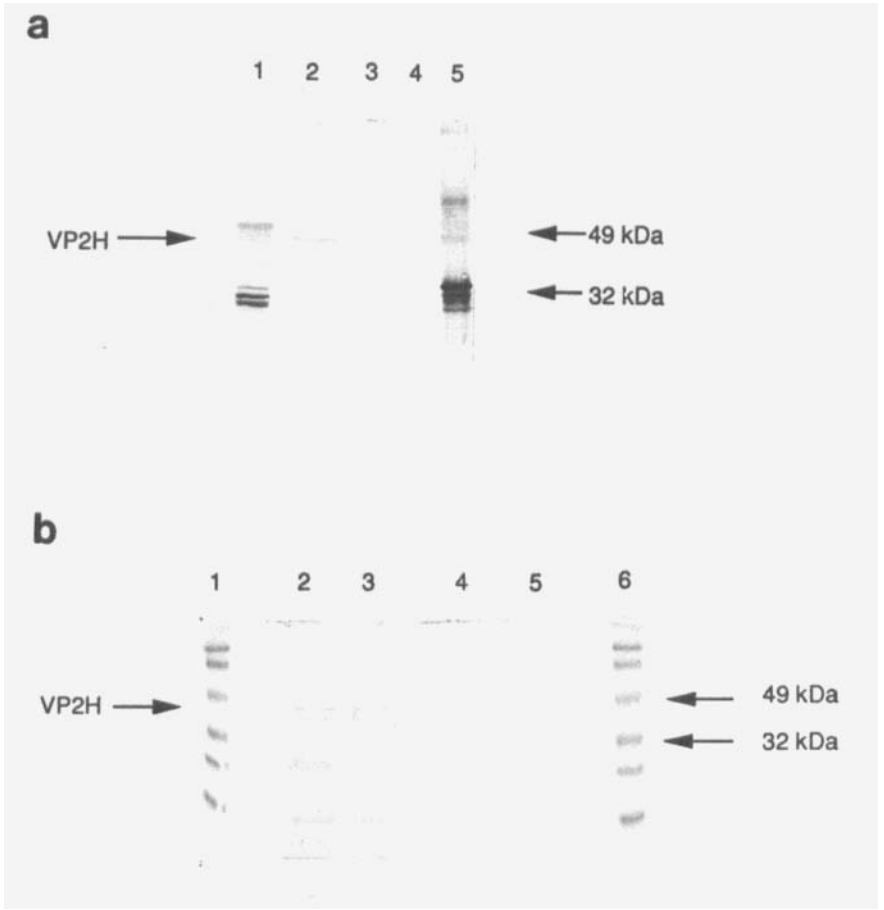

FIGURE 10. a: Western blot analysis of purified VP2H protein from IMAC column. Lane 1, vIBD-7–infected SF-9 cells; lane 2, 10 μL of pooled and concentrated VP2H protein from fractions of 18-26 in FIG. 9; lane 3, uninfected Sf-9 cells; lane 4, AcMNPV-infected Sf-9 cells; lane 5, GLS-5–infected chicken embryo fibroblast (CEF) cell lysates. **b:** SDS-PAGE analysis of purified VP2H protein from IMAC column after Coomassie blue staining. Lanes 1 and 6, molecular weight marker. Lanes 2, 3, 4, and 5 correspond to 10, 7.5, 5, and 2.5 μL of pooled and concentrated VP2H protein from fractions of 18-26 in FIG. 9, respectively. The positions of VP2H and molecular marker (32 kDa and 49 kDa) are indicated.[61]

cells/mL. The time courses of intracellular and extracellular VP2H and VP2 recombinant proteins as shown in FIGURE 11. Intracellular VP2H protein remained low during viral replication. This was also observed for the VP2 protein. However, after 38 hpi, both extracellular VP2H and VP2 protein concentrations increased rapidly. This may indicate that both VP2 and VP2H were liberated due to cell lysis (viability was 43% for VP2H and 41% for VP2 at 38 hpi). The final yields for VP2H and VP2

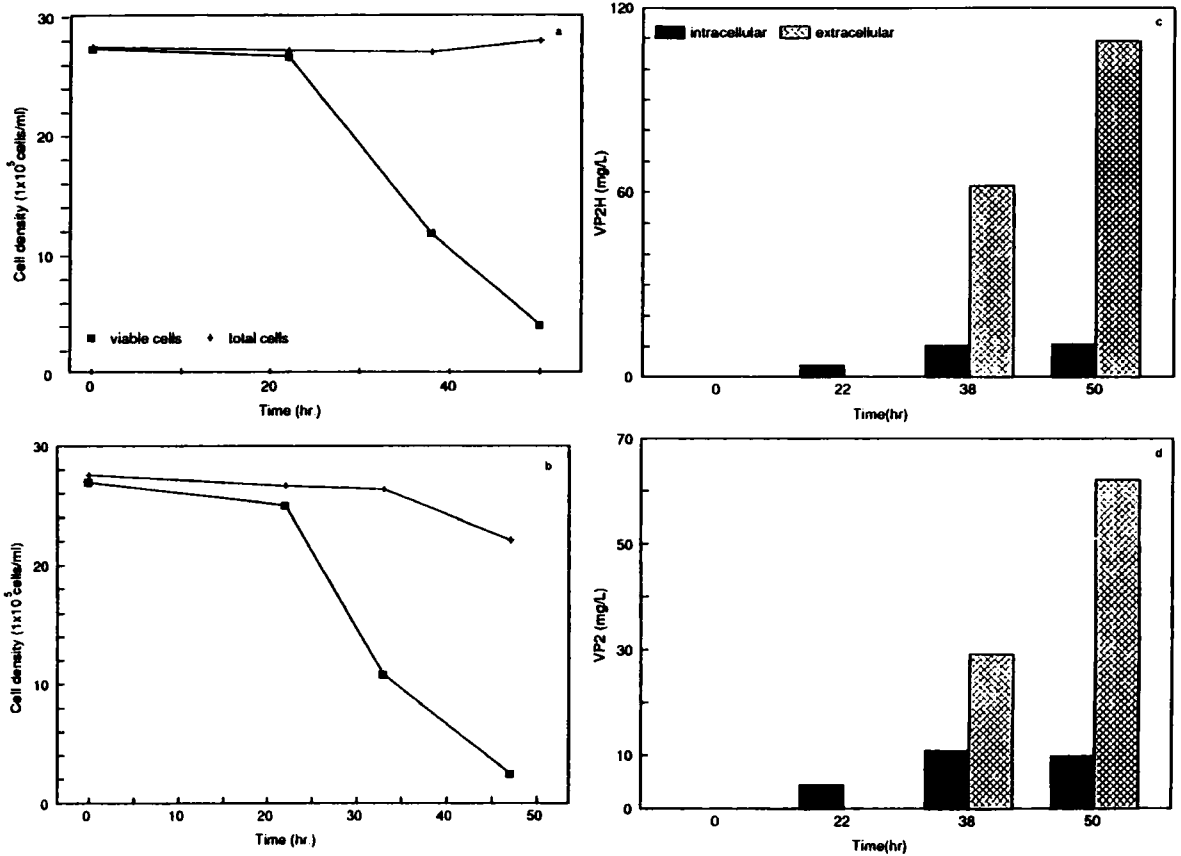

FIGURE 11. Postinfection total and viable cell densities for vEDLH-22 (Fig. 2a, VP2H) and vEDL-8 (Fig. 2b, VP2) baculoviruses. Intracellular and extracellular fractions of VP2H (Fig. 2c) and VP2 (Fig. 2d) are depicted.[61]

proteins were about 120 and 70 mg/L, respectively. Although we have not used IMAC for a large volume purification, we have obtained sufficient data to estimate protein production costs using the bioprocess presented.

CONCLUSIONS

Advantages for the recombinant poultry vaccine, as compared to the injection of an avirulent strain or the transfer of maternal antibody to breeder hens, include potential for complete and passive protection, higher reactivity per milligram of vaccine protein, smaller dose quantities, and more convenient processing. At this point, results on the optimum dose composition and quantity are incomplete, so a direct economic comparison between the processes is unavailable. However, given both the attractiveness of the baculovirus insect cell expression system and the optimization research activity, one might anticipate commercialization of IBDV vaccines and other baculovirus-based products in the near future. The work reviewed and presented in this paper highlights specific areas where further advancements will lead to increased productivity and therefore lower processing cost, namely maintaining the maximum cell number and specific protein productivity, developing convenient and stable bioreactor operating strategies, and integrating unique low-cost separations steps.

ACKNOWLEDGMENTS

We thank Gerard H. Edwards and Sarah Milczanowski for technical assistance.

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