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Estudio del proceso de producción y ensamblaje de proteínas multiméricas a través del sistema células de insecto-baculovirus

T E S I S

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P r e s e n t a

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Nomenclatura

a	MDI bac2	
b	MDI bac6	
ΔTDI	TDI segundo virus - TDI primer virus	h
hpi	horas post-infección	
L_{ARNm}	Longitud del mensajero	nucleótidos
MDI	Multiplicidad de infección	ufp/cel
N_{ARNm}	Número de mensajeros disponibles	
N_{pol}	Número de polimerasas trabajando	
N_{rib}	Número de ribosomas unidos a cada transcrito	
OD	Oxígeno disuelto	%
PF68	Pluronic F68	
pol	polimerasa	
PPV	Pseudopartícula viral	
SCI-B	Sistema células de insecto-baculovirus	
TDI	Tiempo de infección	
U	Unidades	
ufp	Unidades formadoras de placa	
UR	Unidades relativas	
VCO	Velocidad de consumo de oxígeno	mmol O_2/L h
v_{pol}	Velocidad de transcripción	nucleótidos/pol s
v_{rib}	Velocidad de traducción del ribosoma	nucleótidos/ribosoma s
w	Número de partículas virales	
Xt	Concentración celular total	x10^6 cel/mL
Xv	Concentración celular viable	x10^6 cel/mL
y	Número de partículas virales	

Resumen

Una de las ventajas del sistema de expresión de proteínas recombinantes en células de insecto-baculovirus, es la posibilidad de expresar simultáneamente varias proteínas recombinantes que se ensamblan de forma eficiente en multímeros. Esta expresión simultánea puede ser muy compleja, ya que requiere de proteínas diferentes en diferentes cantidades. Uno de los casos más complejos es el de la producción de las pseudopartículas virales (PPV). Las PPV son estructuralmente idénticas al virus nativo, pero carecen del material genético. Si las PPV pudieran ser producidas eficientemente, se convertirían en una alternativa novedosa y eficaz para la inmunización contra varios virus. El diseño de estrategias racionales de producción, que maximizen los rendimientos y la productividad, pueden hacer esto posible. Sin embargo, a pesar de sus ventajas, la información disponible sobre la formación de PPV y sobre estrategias para su producción es aún muy limitada.

Las PPV de rotavirus están formadas por tres capas concéntricas conformadas por las cuatro proteínas más abundantes del rotavirus nativo: VP2, VP6, VP4 y VP7. La presencia de las cuatro proteínas es indispensable para un ensamblaje eficiente de PPV inmunológicamente activas. En células de insecto, las PPV se obtienen a través de la infección simultánea de un cultivo con varios baculovirus recombinantes, que contienen cada uno el o los genes que codifican para las diferentes proteínas recombinantes. En el presente trabajo se pretende lograr un entendimiento integral del proceso de producción de las diferentes proteínas recombinantes y de las PPV por células de insecto. El acercamiento utilizado incluyó desde una caracterización exhaustiva de la producción de cada una de las proteínas recombinantes, hasta su producción en biorreactores totalmente controlados e instrumentados.

Durante la fase de caracterización, las velocidades de producción y las concentraciones máximas de las proteínas individuales fueron función de su peso molecular. Esto es muy importante para el diseño de estrategias de producción. Además, se encontró que la producción simultánea de las proteínas recombinantes no afectó sus velocidades de producción. Por lo tanto, los parámetros cinéticos obtenidos de cultivos que expresan individualmente cada una de las proteínas recombinantes pueden ser extrapolados a los cultivos que producen simultáneamente varias proteínas. Por otro lado, éstos parámetros cinéticos fueron manipulados a través de la multiplicidad de infección (MDI). En cultivos coinfectados, el variar la relación entre las MDI de cada virus, permitió obtener diferentes relaciones estequiométricas entre las proteínas recombinantes. Además, el uso de coinfecciones no simultáneas permitió manipular la velocidad de producción y la concentración de cada proteína recombinante. Estas herramientas son muy útiles para obtener la estequiometría deseada entre las proteínas recombinantes, lo que

permitirá implementar estrategias óptimas de producción. Las cinéticas de formación de PPV de una y dos capas de proteína se siguieron, y el efecto de las diferentes relaciones de proteínas en la concentración intracelular de multímeros fue determinado.

El estudio del efecto del agente protector de esfuerzos de corte, Pluronic F-68 (PF68), en la producción de proteínas y baculovirus recombinantes, permitió ampliar el conocimiento sobre la forma de acción de este aditivo para proporcionar protección contra la agitación. La adición del PF68 incrementó la concentración de proteína recombinante 10 veces, mientras que el título viral máximo obtenido se redujo 20 veces. No se encontraron diferencias entre la reología del medio de cultivo, ni en las cinéticas de crecimiento e infección. Por lo tanto, se determinó que el PF68 tiene un efecto fisiológico sobre las células, lo que descarta el modo de acción puramente físico que ha sido propuesto por algunos grupos de investigación para explicar su efecto protector.

Con el fin de contar con los conocimientos necesarios para diseñar estrategias de alimentación para incrementar la productividad de proteína recombinante, se realizó un estudio del metabolismo celular antes de la infección. Específicamente, se determinó el papel y la interacción entre varios nutrimentos que resultaron ser los más importantes para el crecimiento celular. Se encontró que las velocidades de consumo de la glucosa y la Glutamina están reguladas por la concentración de cada sustrato, aunque la concentración de Glutamina no afectó la velocidad de consumo de glucosa, o viceversa. Un abastecimiento excesivo de glucosa o Glutamina provocó el desperdicio de energía y fuentes de carbono, como lo demostró una reducción en los rendimientos celulares y la acumulación de desechos metabólicos. Se demostró la importancia de un abastecimiento adecuado de nutrimentos, particularmente, la necesidad de mantener las concentraciones de Glutamina y glucosa ligeramente por encima de sus valores críticos.

Finalmente, con base en los resultados anteriores y una caracterización exhaustiva del consumo de nutrimentos después de la infección, se implementó la velocidad de consumo de oxígeno como herramienta para monitorear en línea cambios en el estado fisiológico de las células. Se encontró que el comportamiento dinámico de la velocidad de consumo de oxígeno puede ser correlacionado con el agotamiento de monosacáridos (glucosa y frutosa) y Glutamina. Además, utilizando ésta herramienta fue posible determinar en tiempo real la concentración celular y la eficiencia de la infección.

Abstract

One of the advantages of the widely used insect cell-baculovirus protein expression system is the possibility of simultaneous expression of different proteins which assemble successfully in multimers. Such simultaneous expression can be very complex, as proteins very different in nature may be required at different times in different stoichiometric relations. One of the most complex cases is the production of virus-like particles (VLPs), particularly rotavirus-like particles (RLP), which are structurally identical to native virions, but lack the genomic material. If economically produced, VLPs can become a viable novel immunization alternative for many viruses. Rational production strategies, that maximize yields and productivity, can make this possible. However, in spite of their advantages, information available regarding the formation of VLP and strategies for their production is still very limited.

RLP are formed by three concentric layers of the four most abundant proteins in the native rotavirus: VP2, VP6, VP7 and VP4. The presence of the four proteins is essential to achieve an efficient assembly of immunogenic RLP. In insect cells, RLP are obtained through simultaneous infection of a culture with recombinant baculoviruses, each coding for one or more proteins. In this work, an integral approach for understanding the production of recombinant proteins and RLP by insect cells was pursued. Such an approach spanned from an exhaustive kinetic characterization of the production of each recombinant protein to the production in fully instrumented and controlled reactors.

The individual protein production rates and maximum concentrations were found to be a function of their molecular weight. This has important implications on the design of production strategies. Also, it was found that simultaneous expression of the recombinant proteins did not affect their production rates. Therefore, kinetic parameters obtained in individually infected cultures can be extrapolated to coinfections with various recombinant baculoviruses. By modifying the multiplicity of infection (MOI), we were able to manipulate the protein production rates. By manipulating MOI ratios among viruses in coinfections, different stoichiometric relations between proteins could be obtained. Also, the use of non-simultaneous coinfections was found to allow the manipulation of the production rate and concentration of each recombinant protein. These tools are very useful to achieve a desired protein stoichiometry, which in turn, would allow the design of optimum production strategies. Kinetics of formation of double and single-shelled RLP were followed, and the effect of different protein ratios in intracellular multimer concentrations was determined.

Investigations regarding the effect of the shear protective agent Pluronic F-68 (PF68) on recombinant protein and baculovirus production, allowed a better understanding of the mode of action of this additive. Maximum recombinant VP7 concentration and yield increased 10 times, whereas virus production decreased by 20 times, in spinner flask cultures with 0.05% (w/v) Pluronic F-68 compared to controls lacking the additive. No differences were observed in media rheology, nor kinetics of growth and infection between both cultures. Therefore, PF68 was found to have a physiological effect on insect cells, which discarded an entirely physical mode of action of the additive, as has been proposed by other research groups.

Nutritional information of insect cell cultures, required for designing strategies to increase protein productivity, is still limited. In this work, the role of selected nutrients and their interactions in insect cell metabolism before viral infection were determined. Glucose and Glutamina consumption rates were regulated by their own concentration, although Glutamina concentration did not affect glucose consumption rate or vice versa. Excessive glucose or Glutamina supply caused energy and carbon wastes, as observed by reduced cell yields and accumulation of by-products. The importance of an adequate nutrient supply was demonstrated, particularly, the need to maintain glucose and Glutamina concentrations just above their critical values.

Finally, with an exhaustive characterization of nutrient consumption before and after infection, oxygen uptake rate (OUR) was implemented as a tool to monitor insect cell cultures. The dynamic behavior of OUR could be correlated with monosaccharide (glucose and fructose) and Glutamina. Moreover, using this tool, cell concentration and the effectiveness of infection could be determined on-line and on real-time.

Capítulo I

Introducción

Existe una gran cantidad de productos de interés biotecnológico que están constituidos por multímeros de proteínas. Entre ellos se encuentran los anticuerpos, los virus, la hemoglobina, la insulina, la lactoalbúmina, la hexoquinasa, la triptofano-sintetasa, y otras muchas enzimas. Éstas proteínas oligoméricas pueden incluso estar constituidas por más de 2,000 monómeros de diferentes proteínas, como es el caso de los virus. La producción *in vitro* de éstas proteínas ha despertado un interés creciente, enfocado a lograr un entendimiento del proceso de producción de las subunidades protéicas y de su ensamblaje en multímeros.

Uno de los modelos más interesantes y complejos es el proceso de producción de pseudopartículas virales (PPV), una excelente alternativa para el diseño de vacunas virales. Actualmente, se encuentran en evaluación por la FDA (E.U.A.) vacunas contra cinco diferentes virus basadas en ésta estrategia. De especial interés es el caso de las PPV de rotavirus, ya que más de un millón de personas, principalmente niños, mueren anualmente víctimas del rotavirus. Hasta el momento, la obtención de vacunas efectivas contra éste virus sigue siendo un reto, ya que existen más de 5 diferentes serotipos contra los que la vacuna adecuada tiene que proporcionar protección simultánea. El rotavirus está constituido principalmente por 4 proteínas de tamaños y características distintas, que al ser simultáneamente expresadas por células de insecto conforman PPV estructuralmente idénticas al virus, pero sin el material genético. La producción de vacunas constituidas por PPV polivalentes de rotavirus ha demostrado ser eficiente para la protección simultánea contra varios serotipos (Crawford et al., 1999).

A pesar de las ventajas de las PPV, existe muy poca información en la literatura sobre su producción, aún a pequeña escala. Esto representa un reto único, ya que las 4 diferentes proteínas estructurales deben ser producidas simultáneamente. Además, se requieren diferentes cantidades de cada proteína. Para que las PPV puedan ser utilizadas masivamente es necesario producirlas económicamente en grandes cantidades, y con una composición constante. Esto demanda un sistema de expresión versátil y eficiente. El sistema células de insecto-baculovirus (SCI-B) ha sido el más utilizado para la producción de PPV. Este sistema tiene varias ventajas: es altamente productivo; es un sistema eucariote superior, por lo que es capaz de producir proteínas muy similares a las de mamíferos; y la construcción de nuevos vectores recombinantes es sencilla. Sin embargo, el cultivo *in vitro* de células de insecto tiene limitaciones importantes.

Las células de insecto, al igual que otras células de eucariotes superiores, son muy frágiles, son sensibles a metabolitos generados en los cultivos y tienen requerimientos nutrimentales complejos. Todos estos elementos deben considerarse al producir proteínas recombinantes, ya que la productividad y la calidad del producto dependen directamente del medio ambiente de cultivo. Para lograr una producción adecuada de los multímeros de proteínas, es necesario tener un conocimiento a fondo del proceso. Esto incluye tanto al sistema de expresión en sí, como al proceso de producción de las diferentes proteínas recombinantes y su ensamblaje en multímeros. De esta forma, en este trabajo se realizó un estudio integral del proceso de producción de proteínas multiméricas por el SCI-B, utilizando como modelos la producción de PPV de rotavirus. Esto incluyó el estudio cinético de producción de proteínas recombinantes y PPV , un análisis del metabolismo celular, el desarrollo de estrategias computarizadas de monitoreo y control en biorreactores, y el papel de sustancias protectoras al estrés hidrodinámico (específicamente Pluronic F-68) en la productividad de proteínas y baculovirus recombinantes.

Dentro del cuerpo de ésta tesis se han incluido 4 artículos que se han escrito en el transcurso del trabajo doctoral. Uno de ellos consiste en una revisión de la literatura del estado del arte del SCI-B, y se ha incluído en la sección de antecedentes. La parte de producción de proteínas recombinantes en reactores agitados, que comprende un estudio del papel de sustancias protectoras del estrés hidrodinámico, un estudio del metabolismo de las células de insecto y el uso de la VCO como herramienta para monitorear y controlar cultivos de células de insecto, incluye 3 artículos, 2 de ellos ya publicados y uno sometido. Adicionalmente, durante la realización del presente trabajo se escribieron 2 capítulos (con arbitraje) que formarán parte de la Encyclopedia of Cell Technology, editada por John Wiley and Sons (Palomares y Ramírez, 1999a, 1999b).

Capítulo II

Antecedentes

II.1 El sistema células de insecto-baculovirus

INSECT CELL CULTURE: RECENT ADVANCES, BIOENGINEERING CHALLENGES AND IMPLICATIONS IN PROTEIN PRODUCTION

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Abstract

The insect cell culture-baculovirus expression vector system has become popular for protein production. However, extensive information about the requirements and characteristics of insect cell culture has appeared only in the last few years. Such information and recent advances on insect cell culture technology are reviewed in this article. Special emphasis on engineering issues and specific results of our laboratory with Sf-9 cells are given to illustrate the various aspects discussed.

1. Introduction

The production of proteins for therapeutic applications is becoming increasingly important. Traditionally, such proteins have been obtained from recombinant procariotes or lower eukariotes. However, these organisms are not able to perform the postranslational modifications characteristic of human proteins, such as glycosilation, phosphorilation, acylation, amidation, carboximetilation, isopentilation and others. This processing is essential when proteins are used for human therapy, as it determines the immunogenicity, solubility, circulation half life, specific activity, resistance to proteases and other important *in vivo* properties of the final product [33]. It has been estimated that about half of the major therapeutic proteins under development will require a higher eukaryotic expression system [111]. Such figure represents nearly 60% of the total market of recombinant products and emphasizes the importance of correct postranslational processing. Various protein expression systems have been developed in the last decade for protein production using higher eukaryotic cells. Among these, one of the most widely used is the insect cell-baculovirus expression vector system (IC-BEVS). In this system, proteins are produced by insect cells after infection with a recombinant baculovirus containing the protein gene to be expressed. Insect cells, as other higher eukaryotic cells, are able to perform most postranslational modifications present in human proteins [3, 20, 21, 22, 49, 65, 66, 68, 73, 79, 90]. Furthermore, IC-BEVS is highly efficient as different recombinant proteins can constitute from 5 to 70% of the intracellular protein and volumetric yields as high as 500 mg/L have been obtained [118]. Other advantages of the IC-BEVS include the ease for constructing

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recombinant virus, its safety for humans and mammals, possibility of simultaneous expression of two or more proteins, and its high versatility.

The increasing interest placed in the IC-BEVS can be seen in Figure 1, where the number of cites appearing in the Derwent Biotechnology Abstracts database related to protein production in insect cell culture is shown. As seen, the number of references increased exponentially from 1988 to 1991. After a small decrease in 1992, reports have continued to increase until 1995. Although the number of reports seem to have reached a stationary level, the number of patents registered of insect cell processes have consistently increased. These data confirm the commercial interest that exists for applying processes based on the IC-BEVS technology. The relative attention placed on the IC-BEVS by the academic community, compared to other expression systems, is also high. As determined by Ramírez et al. [101], scientific papers using IC-BEVS represented about 6.5% of all the literature related to recombinant fermentations reported in the main biochemical engineering journals. Moreover, 47% of the papers of recombinant protein expression by higher eukaryotic cells corresponded to IC-BEVS, which places it as the most used eukaryotic expression system.

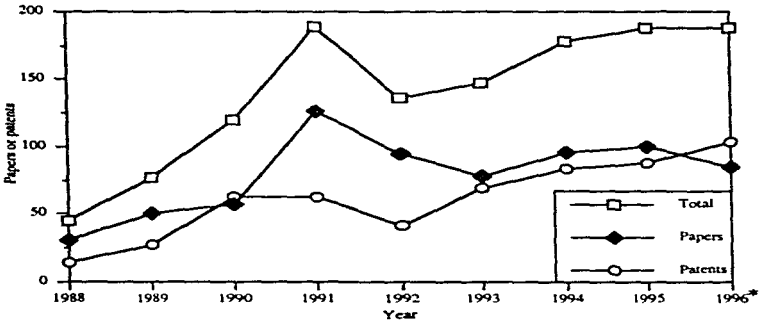

FIGURE 1. Evolution of the number of papers and patents cited in the Derwent Biotechnology Abstracts database about recombinant protein expression in insect cell cultures. *Data for 1996 was extrapolated from the trend of the first 8 months of the year.

Due to its versatility, the IC-BEVS has been used to express over 350 genes from viral, bacterial, fungi, invertebrate, mammalian and plant species [93]. Selected pharmaceutical products that have been successfully expressed in insect cells, and their typical yields are shown in Table 1. Some of the proteins expressed by insect cells are already on clinical trials [125, 144, 145]. Noteworthy is the simultaneous production of several proteins to produce virus-like particles, which are good candidates for vaccine

TABLE 1. Selected pharmaceutical products expressed using IC-BEVS.

Protein	Concentration	Application	Reference
Antistasin	1.7X Y[a] 3.5X MC[a]	anticoagulant and antimetastasic agent	48
Bluetongue virus VLP[b]	-	vaccine	39
Cottontail rabbit papilloma virus VLP[b]	-	vaccine	17
Erythropoietin	8,000 U/mL	anemia	139
Glucocerebrosidase	2.4 μg/mL	Gaucher's disease	107
Granulocyte-colony stimulating factor (G-CSF)	1μg/mL	chemotherapy induced neutropenia	78
Granulocyte-macrophage colony stimulating factor (GM-CSF)	45 μg/mL	infection related to bone marrow transplant	16
Hepatitis B virus antigenic proteins[c]	0.6μg/mg insect larvae	vaccine	98
Herpes simplex virus VLP[b]	-	vaccine	124
HIV VLP[b]	-	vaccine	144
Human papilloma virus VLP[b]	-	vaccine	59
Human parvovirus B19 VLP[b]	35 μg/mL	vaccine	125
Human rotavirus VP6	350 μg/mL	vaccine	10
Influenza virus antigenic protein[c]	11.2μg/mg insect larvae	vaccine	98
α-interferon	-	genital warts	76
β-interferon	50 μg/mL	multiple sclerosis	81
Interleukin-2	-	cancer therapy	116
Interleukin-5	2.5 μg/mL	therapy of autoimmune disease and cancer	46
Nerve growth factor	20 μg/mL	neurological diseases	89
Poliovirus VLP[b]	-	vaccine	126
T-cell growth factor	-	therapy of immune compromised patients	105
Tissue plasminogen activator	2.6 μg/mL	acute myocardial infarction	42
Tumor necrosis factor	70 μg/10^6 cell	tumor regression activity	13

a. Y refers to yeast and MC refers to mammalian cells.
b. VLP indicates "virus like particle", and is formed by all the viral structural proteins without the genetic material.
c. Production by rearing complete insect larvae is included for comparison.

development and excellent tools for the study of protein-protein interactions. Besides the production of recombinant proteins, insect cells have also been used for the production of bioinsecticides, as wild-type baculoviruses are pathogenic to most lepidopteran which are harmful to agriculture.

2. Life cycle of baculoviruses

Baculoviruses belong to the family Baculoviridae, which is characterized by a large, double stranded, circular DNA genome packed in a rod-shaped capsid and further enveloped by a unit membrane [82]. The Baculoviridae family has two genera, the granulosis viruses and the nuclear polyhedrosis viruses, commonly used in IC-BEVS. Baculoviruses have only arthropod hosts with a relatively narrow range. They are naturally protected form harsh environmental factors by a crystalline protein matrix, called polyhedra, constituted by the protein polyhedrin Such viruses are called occluded viruses. When an occluded virus is ingested by insect larvae, polyhedrin is solubilized in the larvae's midgut, and viruses are released. Infection then proceeds. The life cycle of wild type baculovirus in cell culture is summarized in Figure 2. Since culture conditions are not adequate for polyhedra solubilization, cell cultures are infected by non-occluded budded virus (primary infection). During the late infection phase (10-20 hours post infection), new budded virus are produced, which in turn infect other cells (secondary infection). At 20 hours post infection (hpi), polyhedra occluded virus begin to appear (very late infection phase). Cell lysis occurs from 72 hpi. For recombinant protein production, the foreign DNA is usually positioned in place of polyhedrin, which is not required for viral protection *in vitro*. The promoter of polyhedrin is very strong: after infection with wild type baculovirus, polyhedrin can constitute up to 50% of the cellular protein.

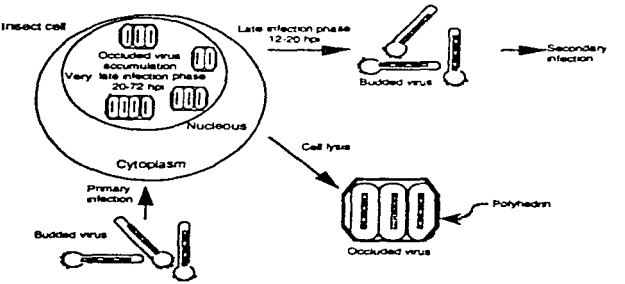

FIGURE 2. Life cycle of wild type baculoviruses cultivated *in vitro*. Insect cells are primarily infected by budded viruses produced in former cultures. At 12 hours post infection (hpi), new viral progeny buds through the cell membrane. After 20 hpi, polyhedrin synthesis begins, and occluded viruses are formed. Occluded virus accumulate in the nucleus, until cellular lysis. Adapted from 8.

3. Intrinsic properties of insect cell lines and viruses.

3.1 INSECT AND VIRAL HOSTS.

Different insect cell lines can exhibit various phenotypes. Some, as ovary derived cell lines, grow weakly attached to suitable surfaces forming a single cell layer. Such cell lines are easily detached from surfaces solely by mechanical means and without the need for trypsinization procedures, and can be grown in suspension without previous adaptation. Other cell lines, as those derived from *Trichoplusia ni*, are anchorage-dependent and exhibit a fibroblastic-like structure. These cell lines have to be adapted to grow in suspension, and are more susceptible to shear stress [31, 108]. Several cell lines have been isolated from insects, usually from lepidopteran and dipteran (see Table 2). The most popular cell lines have been derived from *Spodoptera frugiperda*. Among these is Sf-9, a clone obtained from the pupal ovarian tissue and derived from the previously isolated Sf-21 [116]. Sf-9 is the most widely used cell line for protein production, and β-galactosidase the most commonly expressed protein. Thus, in Table 2 β-galactosidase specific yields of the various cell lines are compared to the yield of Sf-9 in order to assess their relative potential. In Table 3, the yield of other proteins and other cell lines are shown and, where data were available, compared to yields of Sf-9. It can be seen that insect cell lines derived from *Trichoplusia ni* produce up to 20 times more protein than Sf-9. Moreover, high density (8.5×10^6 cell/mL) suspended batch cultures of such cell lines have been obtained [108], particularly of the commercial cell line High Five® (BTI-TN-5B1-4), which has boosted its use.

Another option for recombinant protein expression in insects is the use of live larvae. Certain types of postranslational modifications are performed more efficiently by larvae than by cultured cells, although such modifications can be heterogeneous due to the variety of cells that express and modify the recombinant protein. Also, expression in larvae can be less costly than cell culture, but protein purification can be difficult, expensive and unsuitable for therapeutics. Nonetheless, specific applications may require the use of insect larvae for protein expression.

The most popular baculovirus for the IC-BEVS is *Autographa californica* nuclear polyhedrosis virus, even though there exist reports of more than 500 baculoviruses [93]. This virus has a relatively wide host range, if compared to other baculoviruses, and infect most lepidoterans. Also, different transfer plasmids and parent viruses that allow easy construction of recombinants with different characteristics are already available. Commercial companies, as PharMingen®, have made available baculoviral DNA with lethal deletions that, in combination with suitable vectors, allow easy construction and isolation of recombinants. Vectors with a wide range of characteristics are also available. The recombinant protein gene can be positioned under other promoters besides the one of polyhedrin, such as the very late promoter p-10, or others that allow expression in the late or even in the early infection phases [93]. Earlier expression yields proteins with correct postranslational modifications, as will be discussed below. Vectors that allow the construction of recombinant viruses with multiple genes (up to 4 different proteins can be codified by one recombinant virus) positioned under different promoters are also commercially available.

TABLE 2. Insect cell lines and their volumetric yields of β-galactosidase as compared to the volumetric yield of Sf-9. Adapted from 42 and 137

Cell line	β-gal / β-gal (Sf-9)
Trichoplusia ni Tn M	2.43
Trichoplusia ni BTI-TN-5B1-4 (High Five™)	2.22
Mamestra brassicae M60507	1.44
Trichoplusia ni Tn 368	0.05-1.41
Trichoplusia ni Tn Ap2	1.32
Spodoptera frugiperda Sf-21	1.07
Estigmene acrea BTI-EaA	0.01-1.07
Mamestra brassicae IZDMB0503	0.99
Spodoptera exigua UCR-SE-1	0.60
Lymantria dispat IPLB-LdElta	0.47
Trichoplusia ni IPLB-TN- R	0.41
Heliothis virescens IPLB-HvT1	0.36
Spodoptera exigua UCR-SE-1a	0.26
Spodoptera frugiperda Sf-21AE-15	0.09
Lymantria dispat IPLB-LdEltf	0.09
Spodoptera frugiperda Sf-1254	0.05
Spodoptera frugiperda Sf-21AE	0.05
Anticarsia gemmatalis UFL-AG-286	0.03
Manduca sexta CM-1	0.03
Mamestra brassicae SES-MaBr-4	0.02
Mamestra brassicae SES-MaBr-1	0.01
Plutella xylostella BCIRL-PX2-HNV3	0
Mamestra brassicae NIAS-MaBr-92	0
Christoneura fumiferana IPRL CFI	0
Heliothis zea	0

TABLE 3. Other cell lines and diverse protein yields, as compared to Sf-9. Adapted from 13, 19, 85 and 147.

Cell line	Protein	Protein/10^6 cell	Yp/Yp Sf-9
Trichoplusia ni BTI-TN-5B1-4 (High Five®)	SEAP	44.6 U	23.47
Trichoplusia ni BTI-EA-88	SEAP	4.6 U	2.43
Trichoplusia ni BTI-TN-5B1-4 (High Five®)	TNF	65 µg	2.03
Trichoplusia ni Tn 368	SEAP	3.3 U	1.74
Trichoplusia ni IPLB-TN- MG-1	SEAP	3 U	1.58
Mamestra brassicae MB0503	SEAP	2.7 U	1.43
Spodoptera frugiperda Sf-21	SEAP	2.2 U	1.16
Trichoplusia ni BTI-TN-AP2	SEAP	1.1 U	0.58
Mamestra brassicae IZD-MB-0503	TNF	10 µg	0.31
Spodoptera frugiperda Sf-21	TNF	5 µg	0.16
Heliothis zea	TNF	5 µg	0.16
Antherea moth ovarian cells	TNF	5 µg	0.16
Bombyx mori BM-5	CAT	90 µg	N.A.
Bombyx mori BMN-4	CAT	0 µg	0
Helicoverpa zae Hz163	CAT	0 µg	0
Drosophila melanogaster Schneider cells DM	CAT	0 µg	0

SEAP: Secreted alkaline phosphatase, TNF: Tumor necrosis factor, CAT: Chloramphenicol acetyl-transferase. Yield for Sf-9 correspond to values in the same report. N.A. Not available.

Other viruses, such as *Bombyx mori* nuclear polyhedrosis virus (BmNPV) and *Anticarsia gemmatalis* nuclear polyhedrosis virus, have been scarcely studied and the availability of engineered parent DNA or transfer vectors is limited. These viruses have a reduced host range and have been used for expression in particular cell lines [131, 146, 147]. BmNPV can have several advantages when protein expression in insect larvae is desired, as the larvae of *Bombyx mori* has been routinely grown for silk production and is easier to handle than larvae of *Spodoptera frugiperda* [93].

3.2. POSTRANSLATIONAL PROCESSING BY INSECT CELLS

As mentioned before, correct postranslational processing is an essential requirement for therapeutics production. Inadequate postranslational processing derives in non-active proteins, and is the main reason for choosing a higher eukaryotic expression system. Divergent information has appeared regarding the capacity of insect cells to perform postranslational processing. It is known that insect cells have all the enzymes

required to execute postranslational processing equivalent to mammalian cells, such as glycosilation (including the addition of syalic acid) [3, 20, 21, 22, 49, 65, 73, 79, 90], acylation, phosphorilation, palmitation and oligomerization [66, 68]. However, proteins with heterogeneous [21, 22, 26, 65, 80], or incorrect processing [64, 75] have been reported. The inability of insect cells to perform postranslational processing efficiently, even when they have the required cellular machinery, has been attributed to different factors. Among these is the use of a very late promoter, as the one for polyhedrin. Since baculoviral infection is lytic, the cells die before they are able to modify the expressed protein [49]. Also, it has been reported that the polyhedrin promoter is so strong that it arrests the synthesis of any other protein, including the enzymes required for postranslational processing. Correct postranslational processing has been achieved using earlier and weaker promoters [15]. Moreover, different hosts, including insect larvae, have different capabilities for performing postranslational modifications [20, 90]. Likewise, different proteins can be processed more or less efficiently [50].

Another important factor that can be easily overlooked is the influence of environmental culture conditions on postranslational processing. Davis and Wood [20] and Davidson and Castellino [22] have addressed this issue in insect cell cultures, although the adequate production conditions are yet to be defined. Several authors have reported the influence of environmental conditions, such as the absence of nutrients or presence of toxic by-products, on the capacity of mammalian cells to perform postranslational processing [104, 120, 132, reviewed in 32]. In insect cell culture, for instance, it has been reported that ammonia can interfere with protein synthesis, postranslational processing and secretion [9, 28, 32, 145]. Further research in this field is needed in order to determine if other conditions, such as pH, dissolved oxygen or temperature, can also affect protein processing.

4. Environmental factors relevant to protein production in insect cells

4.1 SHEAR STRESS AND CELLULAR FRAGILITY

Cellular fragility of animal cells difficults scale-up, as adequate mixing and oxygen supply expose cultures to high shear stresses. Insect cells are relatively tolerant to agitation during growth and viral infection [58, 123]. However, they are very sensitive to stress generated while sparging or by bubble entrainment during vigorous agitation [5, 14, 58, 86, 87]. It has been reported that sensitive insect cells are affected by shear stress above 0.1 N/m^2 [31]. Nevertheless, cell fragility studies should consider both, the magnitude of shear stress as well as the time of exposure, something that is usually omitted. Al-Rubeai et al. [2] have reported that cells in the S or G2 phases of the cell cycle are more fragile than in other phases. This is important since cells in the late or middle S phase have been reported to be more susceptible to viral infection [128]. Thus, a culture subjected to high shear stress would be poorly infected if cells in the S phase die. Moreover, infected cells have been reported to be more sensitive to shear stress than uninfected cells, and even if viability is not affected by shear stress, specific protein productivity can be reduced [58, 87].

An effective solution to the problem of cell fragility has been the addition of the non-ionic copolymer Pluronic F-68® [87]. As shown in Figure 3, very low concentrations of Pluronic F-68® can protect insect cells from vigorous agitation, as has also been observed by others [31, 70, 86, 87]. Two mechanisms of protection by Pluronic F-68®

have been reported. On one hand, Pluronic F-68® lowers the culture medium surface tension. This impedes the attachment of cells to bubbles, which liberate lethal energy during bursting [5, 31]. On the other hand, Ramírez and Mutharasan [103] have shown that Pluronic F-68® interacts with the cell membrane, increasing its rigidity and making it more resistant to hydrodynamic forces. The addition of Pluronic F-68® has allowed the culture of insect cells even in sparged or air-lift bioreactors [86]. For other animal cells, an additional effect of Pluronic F-68® include an increased nutrient transport [84] and increased growth rate of static cultures not subjected to shear stress [58]. As discussed below, other strategies to protect cells from deleterious shear stresses include the design of novel impellers, bioreactors and aeration systems.

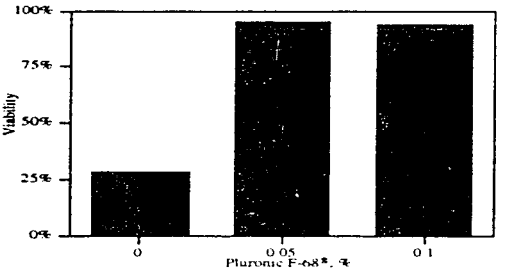

FIGURE 3. Pluronic F-68® protects insect cells from hydrodynamic damage. Sf-9 cells in TNM-FH medium supplemented with 10% fetal bovine serum were vortexed at maximum speed for 30 s. Viability was then measured by trypan blue exclusion.

4.2 DISSOLVED OXYGEN TENSION

Dissolved oxygen tension (DOT) has been generally recognized as a key factor for animal cell growth. Due to cellular fragility, vigorous mixing and sparging is avoided in large scale cultures, resulting in deficient mass transfer and DOT gradients. In turn, such conditions can affect cell growth and metabolism. Moreover, adequate oxygen supply is an essential prerequisite for effective protein expression, as it can increase up to 25 times protein productivity [74, 107, 114, 125, 134, 135]. In contrast, oxygen limitation or excess can induce proteases synthesis and the corresponding degradation of the product of interest [135].

In spite of its importance, quantitative information in literature about the effect of DOT on insect cell growth and protein production is very limited. Most data available are based on the assumption of oxygen limitation in cultures where DOT is neither controlled nor monitored [25, 107, 114, 125], and thus, the information obtained is merely qualitative. The scarce data available has been summarized in Figure 4, where

the effect of DOT on maximum specific cell growth rate is shown. The continuous line in Figure 4 represents the Monod model-fit using the constants calculated by Palomares and Ramírez [96], who obtained full quantitative information of the effect of DOT on cell growth. As seen, the model and constants used provide a good representation of the data reported in literature. It can be seen that critical DOT values occur only below 2% (with respect to air saturation). Furthermore, no effect on nutrient consumption or protein production rates has been observed in cultures at DOT above 2% [38, 52, 96, 135]. Surprisingly, some authors have observed a decrease on cell growth and protein concentration at DOT below 40% [25, 48, 60]. Such behavior also contrasts with data reported for other animal cells [94]. The reduction in protein concentration might be due to the presence of proteases. However, no further reports on this important issue exist.

Although oxygen limitation is one of the most common problems, oxygen-derived free radicals present at high DOT can also harm the cells. Again, contrasting information has been reported regarding the upper limit of DOT innocuous to insect cells. While inhibition has been observed in cultures at or above DOT saturation [25, 48], others have not observed inhibition up to 100% DOT [38]. Further studies are still needed to obtain conclusive data.

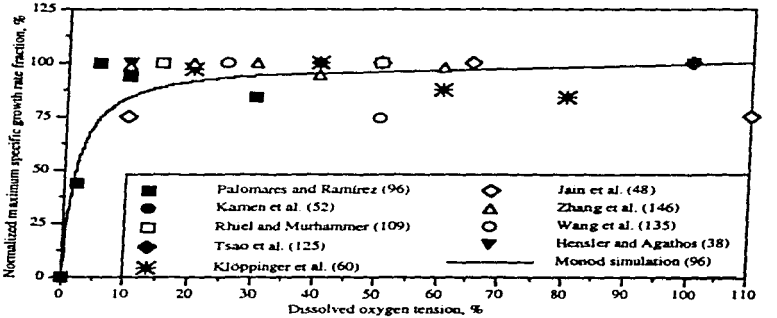

FIGURE 4. Effect of DOT on maximum cell concentration and specific growth rate of insect cells. Data was normalized with respect to the maximum value of each report. Continuous line represents Monod model simulation, where K_{O_2} is 1.91% and μ_{max} is 0.033 h^{-1}, as determined by Palomares and Ramírez [96].

4.3 pH, OSMOLARITY AND TEMPERATURE.

The optimal pH for insect cell growth has been determined to be between 6.2 and 6.3 [41, 62, 143, 146], although some cell lines require a pH slightly higher [110]. In Figure 5, the evolution of pH in a batch bioreactor culture is shown. pH remains fairly constant

until the onset of the stationary phase. Accordingly, pH in insect cell cultures is usually not controlled. From the start of the stationary phase, pH increased consistently. Such a behavior can be explained by the consumption of lactate after glucose depletion. Controlling pH during this phase has increased the cellular concentration [110], and is important for correct protein postranslational processing [146, 147].

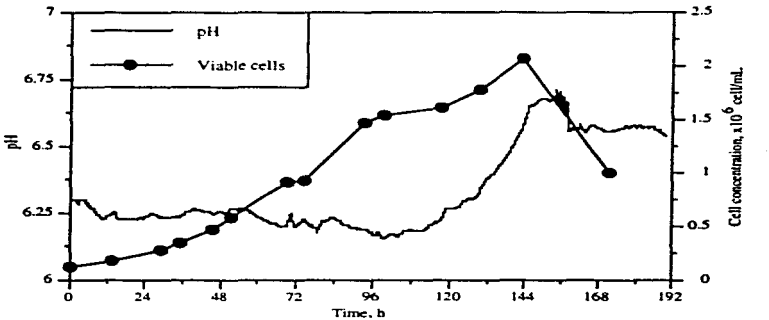

FIGURE 5. Typical pH profile of insect cell cultures grown in instrumented bioreactors. Cells were grown in a 1L bioreactor in TNM-FH medium supplemented with 10% fetal bovine serum.

Insect cells can tolerate a wider range of osmolarity than mammalian cells. While optimal osmolarity for mammalian cell culture extend between 280 to 320 mOsm, insect cells can tolerate from 300 to 380 mOsm [45, 47, 112, 136, 146]. However, Yang et al. [145] have reported a significant reduction in protein production by an increase of 30 mOsm. This contrasts with the response of hybridomas to osmotic stress, which has been reported to improve the specific monoclonal antibody productivity [95]. The evolution of osmolarity in insect cell culture in a batch reactor is shown in Figure 6, which to our knowledge is the only information available in the literature. It can be seen that osmolarity remained constant during exponential cell growth, but later increased during the stationary phase and drastically declined during the death phase. Changes in the osmolarity profile coincided with the depletion of glucose and the on-set of fructose and lactate consumption (data not shown). More drastic changes in osmolarity are to be expected in infected cultures, due to cellular lysis. Thus, monitoring and controlling osmolarity during the protein production phase must not be overlooked.

It is known that the temperature required for optimal growth of insect cells is not necessarily the same that for optimal protein production, and particular cases should be studied individually [128]. For instance, a narrow temperature range (27 to 29°C) has been reported for optimal growth, whereas a broader range (22 to 29°C) has been reported for optimal protein production [37, 107, 125]. Likewise, while the highest

protein production rate has been obtained at 27°C, maximum protein concentration was attained at 30°C. Low temperatures during the production phase can result in lower production rates, nevertheless this may allow better postranslational processing. Also, low temperatures may be preferred for production if the expressed protein is susceptible to degradation at higher temperatures. Moreover, the low temperature at which insect cells are cultured, compared to mammalian cells, allow the expression of a temperature sensitive allele of a gene, not possible at 37°C [93].

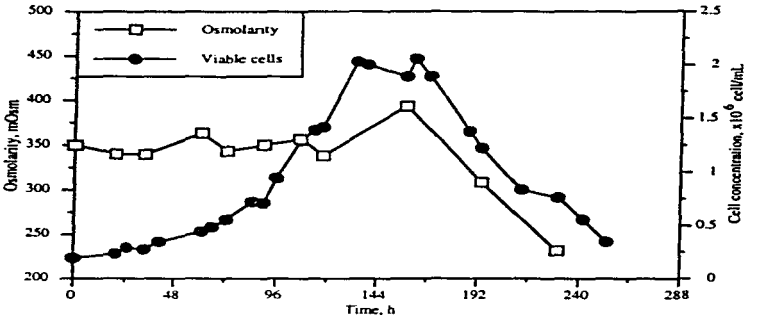

FIGURE 6. Typical osmolarity profile of insect cell cultures grown in instrumented bioreactors. Cells were grown in a 1L bioreactor in TNM-FH medium supplemented with 10% fetal bovine serum.

5. Medium design and nutritional requirements of insect cells cultured *in vitro*

The nutritional requirements of established insect cell lines have been studied only until recently. Consumption rates of key nutrients have been determined, and metabolic pathways have been proposed [7, 27, 30, 88, 92, 96]. However, available information is still incomplete, specially regarding the requirements of infected cells. This has limited medium design and culture strategies to empirical knowledge. The first logical source of this knowledge was the composition of insect hemolymph, which was the basis for traditional media formulation [143].

Insect cells grown *in vitro* require the addition of growth factors, lipids, hormones, and trace elements. Animal serum, usually fetal bovine (FBS), has been added to culture media to provide such factors as well as for shear protection [51]. Insect cells cannot grow without FBS or without the substitutes that have been designed to replace it. The effect of FBS concentration on maximum specific growth rate and maximum viable cell concentration in static cultures is shown in Figure 7. It can be seen that even 0.5% FBS

is not enough to promote growth, and the maximum viable cell concentration almost duplicates when FBS is increased from 5% to 10%. In addition to FBS, lactoalbumin and yestolate have been supplemented by Hink [43] to further improve growth rates and yields. In particular, yeastolate provides vitamins and nucleotides necessary for growth [30].

Serum is the most expensive component of culture media, representing 46% of the cost of TNM-FH medium when supplemented at 10% FBS. Furthermore, undesirable enzymatic activity, lot-to-lot variations, and increased contamination risks, have driven efforts to design serum-free media. Likewise, the high protein content in media supplemented with either serum, lactoalbumin and yeastolate complicate down-stream processing of the desired protein. To overcome this problem, low protein media have been recently developed. In Table 4, the most commonly used media for insect cell culture and their cost are listed. It can be seen that serum-free media have a lower cost than traditional media, and higher cell growth rates and concentrations can be achieved. In addition, lot-to-lot variations of serum are eliminated and down-stream processing is facilitated.

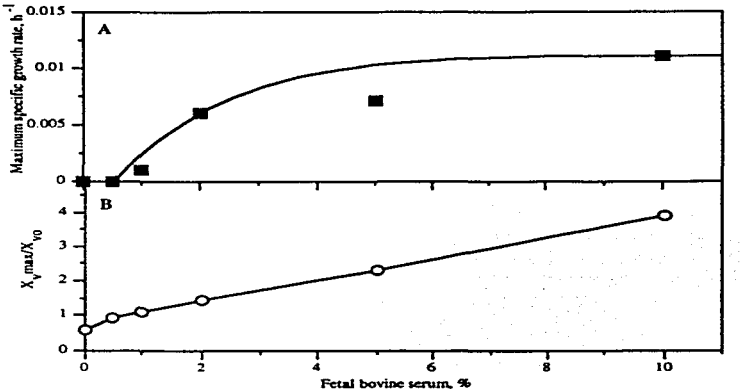

FIGURE 7. The effect of fetal bovine serum in insect cell growth. A. Maximum specific growth rate. B. Ratio of maximum to initial cell concentration.

Monosaccharides, such as glucose and fructose, are the main energy source for insect cells, and their exhaustion results in growth cessation [7, 27, 88, 96]. Neermann and

Wagner [88] found that almost 60% of the glucose consumed by insect cells is degraded to CO_2, and the remaining 40% is used for biosynthesis. Also, from their data it can be calculated that glutamine provides only 8% of the total energy of the cells, although some authors have considered it as the main energy source [117]. Moreover, if some nutrient is in limiting concentrations, including oxygen, cell metabolism may change and other nutrients, as glutamine, can become important energy sources [92, 96]. Further information about metabolic flows can be found elsewhere [7, 30, 92].

TABLE 4 Most commonly used culture media for insect cells

Medium	Xv max $x10^6$ cell/mL	μmax h^{-1}	Price USD/L	Reference
Ex-cell 405 SFM*	9	0.037	> 40	145
Express five SFM*	8.6	0.028	38.50	108
Sf900II SFM	8.1	0.033	43.00	108
Sf900 SFM	7.9	0.027	80.77	110
IPL-41 10% FBS	6	0.02	64.60	27
Ex-Cell 401 SFM	3.8	0.022	34.10	108
Ex-Cell 400 SFM	3	0.039	34.10	89
TC-100 5% FBS	3	0.027	47.90	58
TNM-FH+10%FBS	3	0.03	72.90	27

* High-five® cells

Amino acids, lipids and vitamins are also important nutrients for insect cell growth. Asn, Met, Asp, Ser, Leu, Cys and Gln are the amino acids with the highest consumption rates [7, 30, 121, 145]. Iso, Val, Phe, Gly, Arg, Ser, His and Tyr are also essential for cell growth [83]. Insect cells require sterols, linoleic and linolenic acid for growth, which must be supplied when serum-free medium is used [34].

Nutritional requirements of insect cells after viral infection have been generally considered to remain unchanged, though they may vary depending on the cell line and the recombinant protein to be produced [54, 99, 106, 133, 134, 135, 140, 145, 147]. However, only few groups have studied in detail nutrient kinetics after infection. Among these, Wong et al. [140] reported an increase in the specific consumption rate of certain amino acids, in some cases up to one order of magnitude, while glucose and glutamine consumption rates remained unchanged. Further research on culture requirements after infection is required since information available is still incomplete or ambiguous.

6. Approaches to culture strategies of insect cells and protein production.

Protein production by insect cell culture is a challenge for process engineering. In a first stage, healthy cells have to be grown to the desired cell density and later infected by a

recombinant baculovirus in order to initiate the protein production phase. Both stages, with contrasting characteristics and nature, must be fully understood to optimize protein production. In Table 5 different culture methods for insect cells are compared. Static and suspended cultures in spinner flasks and shakers have been the systems of choice for protein production in laboratory scale. Static cultures yield low cell densities with low viabilities. However, the required infrastructure is minimal. Cultures from 10 to 1000 mL are usually performed in spinner flasks, although cultures are not usually monitored or controlled. Bioreactor cultures are used from bench-top to large-scale protein production, and will be discussed in the following sections.

TABLE 5. Comparison between different culture strategies used for protein production in Sf-9 insect cells

Bioreactor	Culture medium	Xv_{max} $x10^6$ cell/mL	μ_{max} h^{-1}	Scalable	Mass transfer concerns
Static cultures	TNM-FH[a]	1.27	0.02	No	Yes
Spinner flasks (100 mL)	TNM-FH[a]	1.67	0.025	< 5L	Some
Stirred tank	TNM-FH[a]	2.8	0.033	Yes	No
Air-lift [56]	Sf 900[b]	10	0.029	Yes	No
Perfused [10]	TNM-FH[a]	15	0.015	Yes	No
Packed bed [4]	IPL41[b]	2.5	0.008	radially	axially
Silicone membrane tubing aerated [60]	TC 100[a]	2.3	0.032	limited	No
Microcapsule entrapped cells [55]	TC100[a]	80[d]	N.A.	Yes	Yes
HARV[c] [18]	EXCELL 401[b]	14	0.017	No	No

a. 10% FBS. b. serum-free medium. c. High aspect rotating wall vessel. d. per microcapsule volume.

6.1 BATCH AND FED-BATCH CULTURES.

In order to make the IC-BEVS competitive with other expression systems, production costs must be reduced, and productivity increased. This can be accomplished by the development of rational culture strategies which require a full understanding of insect cell metabolism, before and after viral infection. As such knowledge was not available, the first process design approaches were based on information acquired from mammalian cell culture. Accordingly, glucose and glutamine were considered as the key nutrients for insect cell growth and protein production, and ammonia and lactate, metabolites produced in high quantity by mammalian cells, were considered as toxic by-products to insect cells [7, 30, 35, 54, 83, 100, 106, 110, 117, 133, 134]. Considering that a main source for ammonia and lactate generation is a high glutamine and/or glucose concentration, efforts were focused on controlling such nutrients at low, but non-limiting levels. However, glutamine and/or glucose feeding has failed to increase significantly the cell concentration or protein productivity of cultures [10, 30, 70, 89, 110]. Further research demonstrated that insect cell metabolism has substantial differences compared to many mammalian cells in culture. For instance, glutamine is not essential for insect cell growth, as they can be produce it from glutamate and ammonia [91]. Moreover, lactate and ammonia are not produced by cultures with an adequate nutrient and oxygen supply [7, 27, 54, 92, 96, 109, 134, 135], and even if such

by-products were accumulated in the culture broth, concentrations of up to 15 mM do not inhibit insect cell growth [96]. Interestingly, both by-products are consumed by insect cells, lactate for pyruvate synthesis during low monosaccharide concentration, and ammonia for glutamine synthesis during glutamine limitation [54, 92, 96, 117, 134].

After viral infection, adequate nutrient supply has a two-fold importance: maintain culture viability and provide substrates for protein production. Specific protein productivity is reduced if carbon and energy sources are not available [6, 38]. Nonetheless, the generation of ammonia due to nutrient excess is specially relevant during this phase, since it can affect the capacity of insect cells to perform postranslational modifications even without affecting growth [9, 28, 32, 145]. Also, lactate can reduce viral infectivity and thus recombinant protein productivity [125, 142]. Moreover, if protein production strategies that allow cell growth after viral infection are used, then nutrient supply needed for further cell growth must be ensured.

To date, feeding of complex mixtures of nutrients, in addition to glucose and glutamine, has yielded better results than individual nutrient feeding. Among the substances added with good results are FBS [70, 110], yeastolate [7, 70, 89, 106] and a mixture of amino acids [7]. Complete medium addition has also increased protein concentration [69]. As success with nutrient feeding has been partial, complete replacement of culture media before infection has become the strategy of choice to avoid nutrient limitation and eliminate by-products [7, 10, 38, 57, 60, 69, 70, 71, 74, 107, 110, 121, 134, 147]. However, such strategy is difficult to scale-up, and expensive culture medium is inefficiently used. A better understanding of insect cell metabolism is still needed for developing rational feeding strategies.

6.2 CONTINUOUS AND PERFUSION CULTURES.

Protein production by IC-BEVS in a continuous system is only possible by a two stage configuration: one for cell growth and another for cell infection [61, 123, 127, 128, 129, 147]. Steady state has been successfully achieved in continuous culture and specific protein productivity has been similar to spinner flasks. However, productivity decreases as continuous reactors are operated for long periods (i.e. over a month [61]) possibly due to the appearance of defective virus after excessive successive passages in the infection vessel. Defective virus lack up to 40% of the viral genome, including the polyhedrin gene and genes required for viral replication [138]. Defective virus compete with intact virus for cellular proteins and enzymes, and thus interfere with intact virus replication. Additionally, they can replicate faster than intact virus [129, 138]. Defective virus can be detected after passage 10, and their negative effect on protein production is very severe after passage 20 [61]. Accordingly, semicontinuous and repeated batch cultures have been designed to overcome this problem [24, 130].

Perfusion cultures are widely used for many animal cells, however, they have only been used for insect cell cultures by few groups. Incremented cell [11, 25] and polyhedra [60] production have been obtained, although at high costs due to the large volumes of expensive culture media needed. Caron et al. [11] reported a reduction of the maximum growth rate and specific protein production rate during perfusion cultures. Such reductions were caused by cellular damage in the separation device, a tangential filter. The main problem in the operation of perfusion cultures has been the separation of cells from the medium by filters. Electric, sonic or gravitational settlers [119] are some of the proposed alternatives that can solve such a problem. Nonetheless, these

systems are difficult to scale-up. Improvements in this field are needed before perfusion culture can become an adequate strategy for protein production by insect cells.

6.3 CHARACTERISTIC BIOREACTORS AND OPERATION MODES.

Specific oxygen demand of insect cells is similar to mammalian cells [96], however, the maximum cell concentrations are typically much higher in insect cell cultures. Accordingly, overall oxygen demand of insect cell cultures, is 3 to 4 times higher than in mammalian cell cultures grown under similar conditions. As a result, a variety of novel bioreactors, aeration devices and impellers have been designed to satisfy the oxygen demand without imposing deleterious hydrodynamic forces to the fragile insect cells.

Due to the sensitivity of animal cells to sparging, surface aeration is the strategy of choice. Simple modifications, such as placing an impeller at the gas-liquid interface, can increase the system k_La several-fold, and thus, the viable cell concentration [44]. Kamen et al. [54] proposed the use of a helical ribbon impeller which provided sufficient oxygen transfer to an 11 L culture, without any cellular damage. Moreover, some groups [1, 36, 60] have successfully aerated cultures through silicone tubing, although this alternative can be difficult and expensive to scale up. Initial attempts to grow insect cells in air-lift bioreactor failed due to cell death caused by sparging [87, 122, 142]. The problem was overcome by addition of protective agents such as Pluronic F-68®, and cultures up to 21 liters have been conducted with similar cellular and protein productivities than laboratory scale cultures [77, 86]. Air-lift bioreactors have also been used successfully to grow attachment-dependent insect cells in packed beds, reaching product concentrations up to 35% of the total culture protein [63, 115]. Other strategies for protecting insect cells to shear stress include their entrapment in microcapsules [55, 56]. High cell concentrations were achieved by microencapsulation, although specific virus production was lower than in other systems. Microcapsules have the additional advantage of retaining proteins with molecular weight higher than 6 kDa, which can facilitate product recovery.

The two-stage process of protein production in insect cells led naturally to the design of repeated batch cultures in a two stage bioreactor configuration [24, 97, 130, 147]. These cascade systems allow semicontinuous operation for longer periods than continuous processes, with a reduction in the generation of defective particles, and at productivities comparable to batch processes [1, 24, 97, 130, 147]. Only van Lier et al. [130] have reported a reduction of productivity in these systems, which was attributed to oxygen limitation. Further research on the establishment of optimum operation parameters is desirable.

Other reactor designs include a rotating biological disk system, with no success [115], and a high aspect rotating wall vessel (HARV), which simulates microgravity [18]. Insect cell cultures in the HARV had lower growth rates, but high culture viability was prolonged, as the death rate was 40 times lower than the one observed in shaker flasks This was attributed to a reduction of hydrodynamic forces in the microgravity vessel. Although this system is not intended for large scale protein production, it can help understand death mechanisms of insect cells.

6.4 MULTIPLICITY AND TIME OF INFECTION

The multiplicity and time of infection (MOI and TOI, respectively) of insect cell cultures are key factors which determine protein productivity. These two factors interact with each other on defining infection strategies. After viral infection, two very different populations of insect cells are formed: infected and non-infected cells. While non-infected cells continue duplicating, if nutrients and oxygen are available, infected cells do not duplicate. In Figure 8, the typical kinetics of an infected batch culture are shown. For the case illustrated, new budded viral progeny was detected approximately at 18 hpi (dashed-line). The infected cell population further increased as cells not infected initially were infected by secondary or successive infections. From 30 hpi, the onset of recombinant protein production was detected (dotted-line). In this case, the synthesis of both recombinant products (virus and protein) had similar kinetics. In figure 8, the TOI is reported as the percentage of maximum cell concentration at the time of infection. Expressing TOI in such a manner is better than simply as the cell concentration or the time transcurred from inoculation to infection, as is usually done.

It has been shown that cell infection is independent of agitation if the whole cell surface is available for viral contact. The infection process is basically ruled by brownian diffusion [57], and thus, infection of attached cultures can be less effective. The probability, p, of a cell to be infected by w virus follows the Poisson distribution [23, 72, 125], and is dependent on the MOI used, according to the following equation:

$$p(w) = \left(\frac{MOI^{w}}{w!} \right) e^{-MOI} \qquad (1)$$

An early time of infection (early exponential growth phase) is usually combined with a low MOI (0.0001-1 pfu/cell). Under these conditions, only a fraction of the cells is initially infected, while the rest of the culture continues in exponential growth until secondary infection (the population fraction infected by at least one viral particle can be calculated by eq. 1). Low MOI have the advantage of requiring low quantities of virus, which need to be produced in other insect cell cultures. Furthermore, low MOI reduce the probability that both, intact and defective virus infect the same cell. Accordingly, the occurrence of defective particles is prevented since they require an intact virus for replication [138]. However, since the infection progress is slower, the expressed protein remains for a longer period in the bioreactor, increasing its exposure to cellular proteases.

A high TOI (90-100% of the maximum cell concentration) is usually combined with high MOI (5-500 pfu/cell). In this case, cells are infected immediately, and no further cell growth is observed. This strategy requires the addition of large viral stocks, and favors the selection of fast-replicative defective virus [138]. However, specific protein productivity has been reported to decrease when high density cultures are infected at high MOI, compared to similar cultures infected at low MOI [89, 125]. Such a behavior was initially attributed to intracellular interactions that blocked efficient infection [49, 115]. Nonetheless, it has become clear that the reduction in specific productivity is due to nutrient or oxygen limitation, and successful infection of high density cultures is possible if preceded by a complete medium exchange, or complex nutrient feeding, as discussed before [6, 10, 60, 69, 70, 74, 107, 133, 140, 147]. In addition, a higher death

rate when cells are infected by more than one viral particle, as can be concluded from data of Licari and Bailey [71], can explain the low productivities obtained at high MOI. On the other hand, Licari and Bailey [71] observed a two-fold increase in protein productivity when MOI was increased from 10 to 100. According to eq. (1), a MOI higher than 5 pfu/cell infects more than 99% of the culture with at least 1 viral particle. Thus, any changes in the culture behavior above 5 pfu/cell reflects the effect of the infection of a single cell by more than one viral particle. In turn, specific protein productivity can be related to the number of viral particles that infect a single cell.

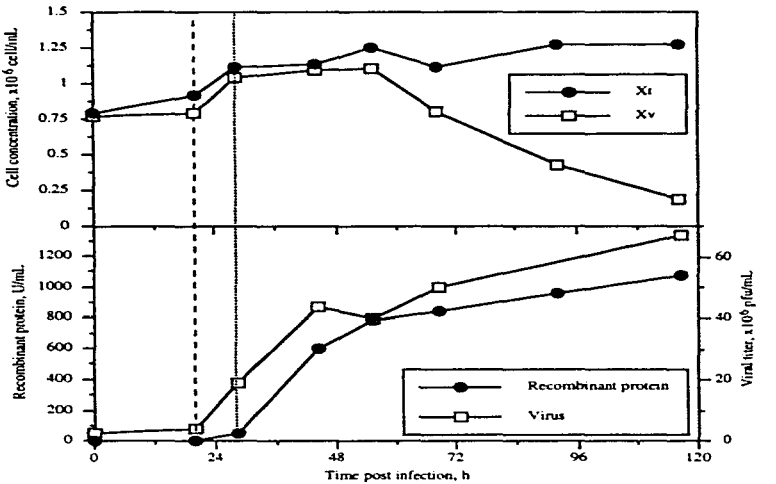

FIGURE 8. Kinetic of recombinant virus and protein production in insect cell cultures. A culture grown in TNM-FH medium with 10% FBS was infected at a TOI of 59% and a MOI of 3 pfu/cell with a recombinant baculovirus that contains the gene of rotaviral VP2 of the strain YM, constructed by the group of Drs. Arias and López in the Institute of Biotechnology of the National University of Mexico. The dotted- and the dashed-lines indicate the time of the onset of recombinant virus and protein production, respectively.

In order to make a rational design of infection strategies, mathematical models have been proposed to calculate an optimal TOI for a desired MOI, or vice versa, to yield maximum protein productivity [99, 141]. For instance, Power et al. [99] have determined the cell growth and infection parameters for infected cultures in Sf900II and

IPL-41 medium. Using such parameters and a set of differential equations, they have been able to predict protein production, viral titers, and culture membrane integrity, in a wide range of TOIs and MOIs. More complex phenomena has been modeled by Tsao et al. [125], who produced virus like particles of parvovirus. Such particles are composed by two different proteins. Thus, infection was carried out with two different virus. By using mathematical modeling, they were able to predict the MOIs at which particles with the correct proportion of the two proteins were obtained, without extensive experimentation. In addition to use the optimal MOI and TOI, cultures to be infected must also have high viabilities, over 90%.

6.4.1 Harvest time.

Baculovirus infection induces the production of proteases not usually produced by non-infected cells. For instance, Wang et al. [135] observed an increase of 15 times in protease activity after 70 hpi. Proteases include those produced by the cells as a defense from viral attack, and viraly encoded proteases expressed during the final phase of infection [71, 93, 135]. Differences on production of various proteins can then be attributed to the differences on their susceptibility to protease degradation. For instance, several authors have not found any significant protein degradation [72, 141], however, this must be determined for individual cases, as some proteins are more stable than others. The harvest time is specially relevant when proteolysis is observed. Harvest time is also an important parameter when virus production is aimed, since viral particles can be inactivated during prolonged exposure to culture conditions [99]. Furthermore, delayed harvest time increases cell lysis and liberation of intracellular proteins, which difficult down stream operations. Thus, an adequate harvest time will increment the productivity of the process. For instance, Tsao et al. [125] recommended to harvest the product when cell viability is between 40 and 70%, as they detected important proteolytic activity after 72 h post infection (hpi).

6.5 MONITORING AND CONTROL STRATEGIES.

Process monitoring and control is a necessary prerequisite for successful scale-up of processes and their further commercial application. Not surprisingly, many groups have concentrated their efforts on the design and development of monitoring and control strategies for animal cell cultures, but only few reports exist for the particular case of IC-BEVS. To date, *in situ* and on-line measurements of a variety of culture parameters and constituents are now possible due to the aid of flow injection analysis (FIA) systems and novel detection sensors based on acoustic resonance, turbidity, culture fluorescence and others. For instance, fluorescence measurements have been used to estimate viable cell density of insect cell cultures even in the death phase, or after viral infection, where most devices fail [1]. Likewise, glutamine has been determined from insect cell culture broths by combining FIA and a chemiluminiscence fiber optic biosensor [12]. Nonetheless, most of the proposed monitoring systems are complicated, non-stable and costly, and thus, have not been incorporated into real time feed-back control algorithms for optimization purposes [40]. A notable exception has been oxygen uptake rate (OUR), which can be monitored on-line by simple inexpensive and stable measurements, such as DO concentration and oxygen composition in the inlet gas stream [25, 29, 38,40, 52, 54, 56, 96, 102, 140].

OUR provides information about the culture metabolic state, as shown in Figure 9. For example, since specific oxygen uptake rate remains constant during the exponential growth phase, the viable cell concentration can be estimated from OUR, as has been also

recently reported for other insect cell cultures [52, 96, 140]. Furthermore, as seen in Figure 9 monosaccharide depletion provokes a reduction in OUR, as has been reported previously [96]. In other animal cell cultures, specific nutrient depletion has been detected and closed-loop feeding strategies based on on-line OUR data have been established [29, 45, 102, 148]. Similar strategies can be used for monitoring and rational nutrient addition in insect cell cultures. Additionally, specific OUR has been reported to increase up to two fold after successful viral infection, and cell lysis can be identified by a reduction of OUR [38, 48, 52, 54, 57, 107, 113, 114, 140]. Similar behavior has been observed for production of porcine virus in mammalian cells, where maximal OUR indicated the time of maximum viral titers [67]. Accordingly, OUR can also be used to monitor the infection phase, and identify the optimum harvest time [52].

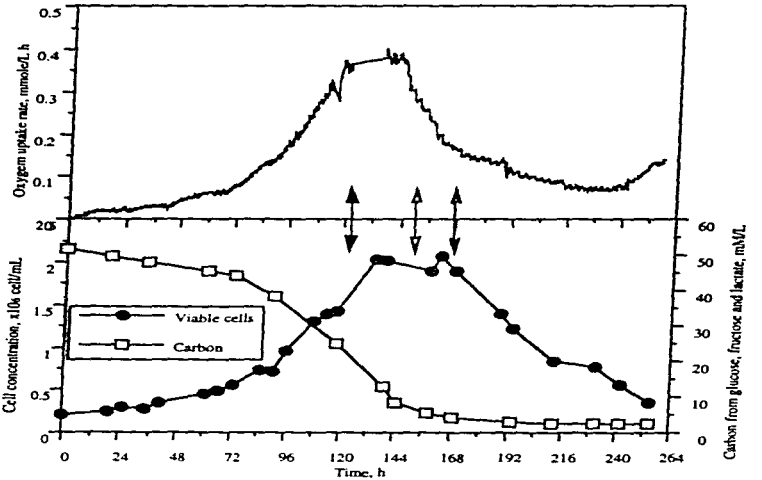

FIGURE 9 On-line oxygen uptake rate estimation using liquid phase balance in a 1 L bioreactor Sf-9 insect cell culture. The arrows indicate glucose depletion (full arrow), fructose depletion (empty arrow) and lactate depletion (line arrow). Carbon concentration was calculated from the sum of the carbon mmoles present from glucose, fructose and lactate.

Traditionally, OUR has been measured on-line by the dynamic method, which has the disadvantage of exposing the cells to undesirable dissolved oxygen fluctuations. Furthermore, the dynamic method provides only discrete data which is not suitable when

fast metabolic events, such as nutrient depletion and viral infection, need to be identified. The limitations of the dynamic method for measuring OUR can be overcome by gas-phase balance methods, although they rely on expensive equipment [53]. Alternatively, the liquid phase balance method can also overcome the limitations of the dynamic method, but with the use of relatively inexpensive equipment [29, 52, 96, 102]. The liquid-phase balance method can provide accurate on-line continuous OUR estimations. Data shown in Figure 9 were estimated using liquid phase balance, and are similar to other data reported in the literature [38, 52, 58, 96, 113, 140]. Other parameters have been successfully used to monitor culture conditions. These include cell size, which increases after infection and is an indicator of successfull infection [48], and CO_2 evolution rate, which closely follows OUR behavior and utility [52]. The development of novel biosensors and their incorporation into feedback control algorithms should further improve the position of the IC-BEVS as a viable commercial technology.

7. Conclusions and future perspectives.

Protein production in insect cells is a challenge to bioprocess engineering, as it is a process with two phases, each with very different characteristics, and where a complex interaction between cell, virus, nutrients and environmental conditions exist. Even when general rules can be defined for the IC-BEVS, it is essential to specifically analyze each protein to be produced. This is specially relevant when highly modified or unstable proteins are to be produced. Protein expression in insect cells is becoming increasingly popular, and the first therapeutics produced by the IC-BEVS may reach the market shortly. The application of bioprocess engineering principles has helped in advancing this field from an empirical art to a more rational and systematic technology. However, further research is still necessary before the IC-BEVS can become a commercial reality.

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II.2 Los rotavirus y las pseudopartículas virales

II.2.1 Nuevos acercamientos al diseño de vacunas virales.

Los virus son causantes de una gran variedad de enfermedades, entre las que destacan la hepatitis, SIDA, poliomielitis, sarampión, rubéola, varicela, herpes, influenza, gripe y la diarrea infantil. Tradicionalmente, las vacunas antivirales han estado basadas en el uso de virus atenuados o inactivados. Sin embargo, estas estrategias tienen la desventaja de la posibilidad de transmisión de las enfermedades por reversión de cepas atenuadas, o por una inactivación deficiente. Para contrarrestar esta desventaja y potenciar la respuesta inmune, se han utilizado nuevas alternativas para la inmunización, que además han proporcionado información importante sobre los mecanismos de patogénesis y la respuesta inmune contra las enfermedades. Entre los nuevos acercamientos están las vacunas con ADN, virus microencapsulados, vectores virales y las pseudopartículas virales (PPV). Las PPV han sido utilizadas con éxito para el desarrollo de vacunas contra rotavirus (Redmond et al., 1993; Crawford et al., 1999), virus de la inmunodeficiencia humana (VIH) (Yamschikov, 1995), virus de la fiebre aftosa (French et al., 1990), parvovirus humano (Tsao et al., 1996) y virus de papiloma humano (Kirnbauer et al., 1993). Algunas de estas se encuentran ya en pruebas clínicas, a punto de ser utilizadas masivamente. Las PPV consisten en la construcción de la cápside viral completa a partir de la coexpresión de cada una de las proteínas virales por organismos recombinantes. Aunque las partículas obtenidas son prácticamente idénticas al virus original y provocan una respuesta inmune similar, no contienen el material genético viral, por lo que no existe el peligro de una infección. Otra de las ventajas de las PPV es la posibilidad de obtención de partículas polivalentes, que contienen proteínas de diferentes serotipos del patógeno, y originan respuesta inmune simultánea contra estos serotipos. A pesar de las ventajas de las PPV, existe muy poca información sobre su producción, aún a pequeña escala. El uso masivo de las PPV dependerá de su producción a bajo costo y con una composición constante.

II.2.2 Los rotavirus y las pseudo partículas virales

Los rotavirus han sido considerados por la O.M.S. como la causa más importante de gastroenteritis viral, por lo que se han redoblado los esfuerzos para ampliar la información sobre la interacción virus-célula huésped y obtener una vacuna eficiente (Estes y Cohen, 1989). Las partículas maduras de rotavirus están formadas por tres capas concéntricas de proteína (**Figura II.1**). La proteína VP2 forma la nucleocápside viral. La cápside interna esta formada por 260 trímeros de la proteína viral VP6. La cápside externa esta formada por VP7,

que tiene una glicosilación alta en manosa ligada a N. La partícula viral posee 60 proyecciones, formadas por dímeros de la proteína viral VP4. Bajo la acción de tripsina, VP4 se hidroliza en dos proteínas, VP5 y VP8 (Mattion et al., 1994). Además de estas proteínas, en la partícula viral nativa existen las proteínas estructurales VP1 y VP3, que se encuentran dentro de la nucleocápside y constituyen entre ambas menos del 3% de la proteína viral total.

Proteína viral	Moléculas/ virión	Peso Molecular	% Proteína total
VP4	120	86 kD	1.5
VP6	780	44 kD	51
VP2	200	102 kD	15
VP7	780	33 kD	30

Figura II.1. Estructura de las PPV de rotavirus (modificado de Mattion et al., 1994).

La morfogénesis de los rotavirus inicia con la formación de partículas subvirales conformadas por doble capa de proteínas (constituidas por VP6, VP2, VP1, VP3 y el ARN viral) en viroplasmas citoplásmicos. Las partículas penetran el retículo endoplásmico, probablemente con ya VP4 asociada a la partícula, donde adquieren la última capa de proteína, constituida por VP7, para conformar partículas virales maduras. Se ha determinado que la formación de las partículas con doble capa de proteínas ocurre muy rápidamente, mientras que la formación de las partículas maduras tiene un retraso de 10 a 15 minutos (Estes, 1996).

Las proteínas de rotavirus se han expresado en células de insecto con el fin de ampliar el conocimiento sobre su función y sobre su interacción con las células a infectar (Labbé et al., 1991; Sabara et al., 1991; Redmond et al., 1993; Crawford et al., 1994; Cuadras, 1998). Las proteínas obtenidas son similares a las proteínas originales, y presentan los mismos epítopes. Crawford et al. (1994) han obtenido PPV de rotavirus a través de coinfectar células de insecto con cuatro baculovirus recombinantes, uno por cada proteína estructural, excepto VP1 y VP3. Estas PPV mantuvieron las características funcionales y estructurales de las partículas virales originales, y se ensamblaron aún en ausencia de VP1, VP3, el ARN viral y las proteínas no estructurales. Estos resultados demuestran que las proteínas estructurales contienen la información intrínseca que se requiere para la formación de partículas (Estes, 1996). Las PPV de rotavirus pueden ser utilizadas para examinar las interacciones entre las proteínas estructurales de rotavirus, el papel de cada proteína estructural en la morfogénesis viral y para generar vacunas.

II.2.3 Estrategias utilizadas para la producción de multímeros de proteínas en células de insecto

En la **Figura II.2** se esquematiza el proceso de producción de PPV de rotavirus. Inicialmente, se cuenta con una pequeña cantidad de células de insecto que deben ser propagadas hasta la densidad celular deseada con la mayor viabilidad posible (>90%). Durante la fase de expansión de las células, el medio ambiente de cultivo y la disponibilidad de nutrimentos son factores clave para lograr obtener una población sana para infectar, como se ha discutido en la **Sección II.1**. A partir del momento de infección se generan dos poblaciones, las de células infectadas y las de células no infectadas. En el caso del sistema de producción de PPV existirán otras subpoblaciones, como aquellas que sólo han sido infectadas por una clase de virus recombinante, las que han sido infectadas por dos, y así sucesivamente. Durante la fase de producción de las proteínas recombinantes, el sistema se complica aún más por la gran cantidad de multímeros que pueden formarse. Además de las PPV completas, se ha observado la producción de otras partículas que carecen de algunas proteínas. La expresión de solamente VP2 en células de insecto genera partículas vacías similares a la nucleocápside, que llamaremos PPV1 (Labbé et al., 1991; Zeng et al., 1992). Crawford et al. (1994) observaron, además, la formación de partículas estables compuestas por las proteínas VP2/VP6, VP2/VP4/VP6, y VP2/VP6/VP7, usando diferentes combinaciones de virus. A estas PPV las llamaremos PPV2, $PPV3_a$ y $PPV3_b$, respectivamente. Al infectar simultáneamente las células con los cuatro virus recombinantes, obtuvieron $PPV3_b$ y partículas con las cuatro proteínas (en adelante referidas como PPV4). Sin embargo, existe la posibilidad de que se formen otras partículas incompletas. Es claro que la formación de la nucleocápside por VP2 es un paso indispensable para la formación de partículas estables (Crawford et al., 1994). Posteriormente, se ensambla VP6, proteína indispensable para la unión de VP7 y VP4. VP4 puede unirse a la pseudo partícula sin necesidad de VP7, y viceversa (Crawford et al., 1994).

La producción de multímeros de proteínas se ha realizado tradicionalmente sin tomar en cuenta las diferencias en el contenido de cada proteína en el complejo final. Las PPV del virus de la fiebre aftosa y de rotavirus se han obtenido a partir de la coinfección de las células de insecto con los mismos MDI y TDI para cada virus, a pesar de que la cantidad de cada una de las proteínas en la partícula viral es diferente (French et al., 1990; Crawford et al., 1994, 1999; Cruz et al., 1998; Jiang et al., 1998). Esto provoca que la célula desperdicie recursos y energía en la producción de proteínas que no serán utilizadas en la formación de la partícula final, o que generarán PPV incompletas.

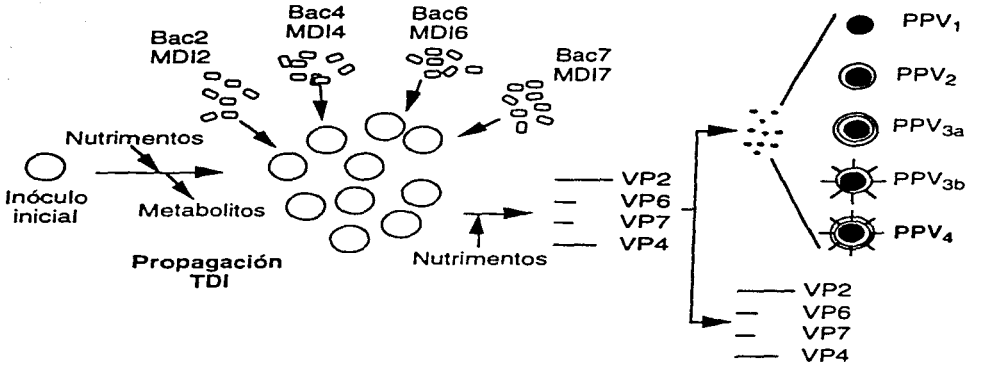

Figura II.2 Esquematización del proceso de producción de PPV de rotavirus en células de insecto. Se ha llamado bac2 al virus BacRf2a, bac4 al virus pVL941/SA11-4, bac6 al virus pAc461/SA11-6 y bac7 al virus pVL942/SA11-9; según nomenclatura propuesta en Crawford et al. (1994, ver **Materiales y Métodos**). Los dibujos no están a escala.

Existen muy pocos estudios que analicen el proceso de producción de PPV, y propongan estrategias para su producción. Jiang et al. (1998) compararon la producción simultánea de las 4 principales proteínas de rotavirus entre las líneas celulares de insecto Sf9 y High Five®. Además, reportaron cinéticas de formación y rendimientos de multímeros, aunque únicamente realizaron una purificación parcial de las PPV (ultracentrifugación en colchón de sacarosa). Es muy probable que otros multímeros, como el propio baculovirus recombinante, estuviera aún presente en las preparaciones de PPV, lo que pudo haber provocado una sobreestimación de la concentración de multímeros. Adicionalmente, la técnica utilizada no permite determinar el tipo o la calidad de las PPV obtenidas. Cruz et al. (1998) realizaron un estudio para determinar las condiciones ambientales adecuadas (velocidades de agitación y aereación y oxígeno disuelto (OD) para la producción de PPV del VIH, que están conformadas únicamente por una proteína Utilizando cromatografía de filtración en gel e inmunodetección, lograron seguir las cinéticas de producción de las PPV y de la proteína no ensamblada. Es muy interesante notar que un cambio en la cantidad de proteína recombinante no necesariamente se vió reflejado en un cambio en la cantidad de PPV, y las mejores condiciones para la producción de las proteínas individuales no son las mismas que para la producción de multímeros. Por ejemplo, la máxima concentración de proteína recombinante se obtuvo a un

OD de 25%, mientras que el máximo ensamblaje se obtuvo a un OD de 10%. Los autores atribuyen éstas diferencias a un posible aumento en proteasas a 25 % OD.

Unicamente el trabajo de Tsao et al. (1990) ha utilizado estrategias de infección para manipular la producción de PPV. Produjeron PPV de parvovirus, las cuales están constituidas por dos proteínas y pueden estar conformadas por diferentes cantidades de cada una. La proporción entre las proteínas determina la inmunogenicidad del producto obtenido. Ya que estas PPV fueron producidas para vacunas fue necesario establecer estrategias de infección que generaran PPV con las proporciones adecuadas entre las proteínas virales. Se encontró que utilizando un baculovirus recombinante para la producción de cada proteína, la MDI de cada virus determina la composición de las PPV obtenidas. Además, la MDI afectó el rendimiento de las PPV. Por lo tanto, es indispensable conocer las condiciones ideales de infección de las células si se desea obtener partículas ensambladas correctamente y en mayores cantidades.

A pesar de la reciente atención que ha recibido la producción y purificación de PPV (Tsao et al., 1996; Jiang et al., 1998; Cruz et al., 1998; Crawford et al., 1999; Hu et al., 1999; Tsoka et al., 1999), aún no existe en la literatura un estudio integral del proceso de producción de PPV. En este trabajo se realizó un estudio de este tipo, que incluye el estudio cinético de producción de cada una de las 4 proteínas recombinantes que conforman las PPV de rotavirus, del ensamblaje de VP2 y VP6 en PPV1 y PPV2, el desarrollo de estrategias de infección para la producción de proteínas recombinantes y PPV, un estudio del metabolismo celular antes de la infección, y propone estrategias de operación adecuadas para la producción de proteínas recombinantes en reactores instrumentados. Dentro de este último punto, se incluye el desarrollo de estrategias computarizadas de monitoreo y control de reactores, y el estudio del papel de protectores de esfuerzos de corte sobre la producción de proteínas y baculovirus recombinantes.

Capítulo III

Objetivos

III.1 Objetivo general

Ampliar el conocimiento sobre los procesos de producción de proteínas multiméricas en general, utilizando como modelo las PPV de rotavirus, con el fin de diseñar estrategias óptimas de producción.

III.2 Objetivos específicos

Obtener información sobre el comportamiento cinético del proceso de producción de proteínas multiméricas. Evaluar parámetros como la velocidad de producción de las proteínas individuales, la MDI, el TDI. Como primer acercamiento y debido a la complejidad del modelo de estudio, únicamente se realizó un estudio del proceso de ensamblaje de VP2 y VP6 en PPV2 y PPV1, las que constituyen la base para la conformación de PPV4.

Obtener información sobre los requerimientos nutrimentales y ambientales de las células de insecto y su papel en el crecimiento celular y producción de proteínas recombinantes. Específicamente, evaluar el papel de los nutrimentos clave y de sustancias protectoras de esfuerzos de corte (PF68).

Desarrollar estrategias de control computarizado del sistema con base en el uso de la estimación en línea de las velocidades de consumo de oxígeno, de consumo de nutrimentos y de generación de metabolitos.

Desarrollar, con base en la información obtenida, estrategias racionales e integrales para la producción de PPV a partir de células de insecto.

Capítulo IV

Materiales y Métodos*

IV.1 Línea Celular

Se utilizó la línea celular Sf-9 (Número de catálogo de la ATCC 1711). Esta línea celular proviene del ovario de pupa del lepidóptero *Spodoptera frugiperda* (conocido trivialmente en México como gusano cogollero del maíz y en Estados Unidos como gusano soldado de otoño). La línea Sf-9 fue clonada en 1983 por Smith et al. (1983), a partir de la línea Sf-21, obtenida por Vaughn et al. (1979) en 1977. Los experimentos se realizaron a partir de dos tipos de células:

A. Células adaptadas al medio de cultivo libre de suero Sf-900II (ver inciso IV.2), donadas en el pase 30 por el grupo del Dr. Amine Kamen (Biotechnology Institute, National Research Council, Canada).

B. Células adaptadas al medio TNM-FH con 10% de suero fetal de bovino, donadas por los Drs. Carlos Arias y Susana López, Instituto de Biotecnología, UNAM. Las células se congelaron en un número de pase desconocido, al que se tomó como pase cero.

Todos los experimentos se realizaron a partir de un mismo lote de células de cada tipo congeladas en nitrógeno líquido. En todos los experimentos, las células se mantuvieron solo hasta un número de pase de 50 después de descongeladas. Las células para inóculo se mantuvieron en cultivo a 27°C en atmósfera sin provisión de CO_2, en frascos agitados a 100 rpm. Todos los inóculos se realizaron con células en fase de crecimiento exponencial, y se inoculó siempre con una concentración celular de 0.2×10^6 cel/mL y con una viabilidad superior al 90%. Con estos procedimiento se asegura que todos los experimentos fueron inoculados con células en las mismas condiciones.

IV.2 Medios de Cultivo

Se utilizaron dos medios de cultivo:

* Algunas de las metodologías utilizadas fueron descritas más ampliamente en los artículos publicados que están incluidos en ésta tesis.

A. Medio con bajo contenido de proteínas y sin suero bovino Sf-900II (GIBCO, Cat. 10902-088). Este medio viene ya en forma líquida listo para usarse. Su composición es confidencial y propiedad del fabricante.

B. Medio TNM-FH (*Trichoplusia ni* medium- Fred Hink). El medio se preparó a partir de medio de Grace (GIBCO, cat. 11300-043) adicionado con 3.33 g/L de hidrolizado de lactoalbúmina (Sigma, cat. L-9010). El medio se disolvió en agua filtrada en un equipo Milli-Q (Millipore Corporation) y se ajustó a pH 6 con hidróxido de sodio 1M. Después de esterilizar el medio por filtración a través de membrana de 0.22 µm, se suplementó con 16.7 mL/L de solución ultrafiltrada de extracto de levadura (concentración final 3.33 g/L, GIBCO, cat. 18200-048), 10% v/v de suero fetal bovino (SFB, GIBCO, cat.16000-044) y con 0.05% p/v de Pluronic F-68 (PF68, BASF). La composición del medio de cultivo varió en algunos casos que se indican en el texto.

IV.3 Baculovirus recombinates

En la **Tabla IV.1** se muestran los baculovirus (virus de polihedrosis nuclear de *Autographa californica*) recombinantes utilizados. Los baculovirus recombinantes fueron donados por el laboratorio de los Drs. Arias y López. Para evitar la aparición de partículas virales defectivas en ningún caso se utilizó un lote de baculovirus recombinante en un pase mayor a 6. Los baculovirus se amplificaron en cultivos con medio TNM-FH 0.5% SFB en frascos agitados.

Tabla IV.1 Baculovirus recombinantes utilizados.

Baculovirus	Proteína recombinante	Cepa de rotavirus	Promotor	Referencia
Bac2	VP2	RF	Polihedrina	Crawford et al., 1994
Bac6	VP6	SA11	Polihedrina	Crawford et al., 1994
Bac7	VP7	SA11	Polihedrina	Crawford et al., 1994
Bac4	VP4	SA11	Polihedrina	Crawford et al., 1994
Bac8	VP8	RRV	Polihedrina	S. López*
Bac2/6	VP2/VP6	YM	p10/p10	Hernández, 1998

*Comunicación personal

IV.4 Cultivos en frascos agitados

Los experimentos se realizaron en frascos agitados de 100 mL, con un volumen de trabajo de 60 mL. Los cultivos se mantuvieron a 27° C y a una agitación de 100 rpm, utilizando un agitador de barra magnética suspendido a aprox. 0.3 cm del fondo del frasco. Los cultivos se infectaron a una densidad de $1x10^6$ cel/mL

IV.5 Cultivos en biorreactor instrumentado

Se utilizaron 2 tipos de biorreactores:

A. Biorreactor instrumentado de 1.7 L con un volumen de trabajo de 700 mL y unicamente aereación superficial con un flujo de 600 mL/min. Para aumentar la transferencia de masa se utilizaron dos impulsores: una barra magnética suspendida con un diámetro del 43% el diámetro de la jarra a 2 cm del fondo y un impulsor de cuatro paletas planas con un diámetro del 57% el diámetro de la jarra a 7 cm de la barra magnética, lo que coincidió con la superficie del líquido. Al impulsor de cuatro paletas se le colocaron dos ampliaciones de lámina de teflón de 3 cm de largo, con el fin de aumentar la transferencia de oxígeno. Además, el reactor cuenta con dos deflectores de 1/10 el diámetro del reactor.

B. Biorreactor instrumentado de 5 L con un volumen de trabajo de 3.8 L (Celligen, New Brunswick Scientific). Este reactor cuenta con un impulsor *cell lift* diseñado por el fabricante. Se utilizó aereación sumergida (0.15 vvm) utilizando el sistema de intercambio de gases del reactor.

Ambos reactores se operaron a 27°C y a una velocidad de agitación de 100 rpm. El oxígeno disuelto se midió con un electrodo polarográfico (Ingold Electrodes). Adicionalmente se midieron el pH y el potencial redox (Ingold Electrodes). La adquisición de los valores de oxígeno disuelto, composición de gases a la entrada, pH y potencial redox se realizó cada 300 s por una computadora Macintosh LC a través de una tarjeta MacADIOS 411 (GW Instruments) equipada con convertidores analógicos/digitales y digitales/analógicos, así como con entradas y salidas digitales. Se utilizó un programa de control escrito en QuickBasic por Aguilar-Aguila et al. (1993). En la **Figura IV.1** se muestra un diagrama de este sistema. La tensión de oxígeno disuelto se controló en un valor constante y predeterminado (30% c.r.sat.aire) a través de la variación de la composición de gases a la entrada (oxígeno y nitrógeno) por medio de un algoritmo de control proporcional, utilizando una ganancia de 2. El flujo de gases se controló con dos controladores de flujo másico 5850E (Brooks Instruments), uno para oxígeno y el otro para nitrógeno (cada uno de ellos con una capacidad de 0-1000 mL/min).

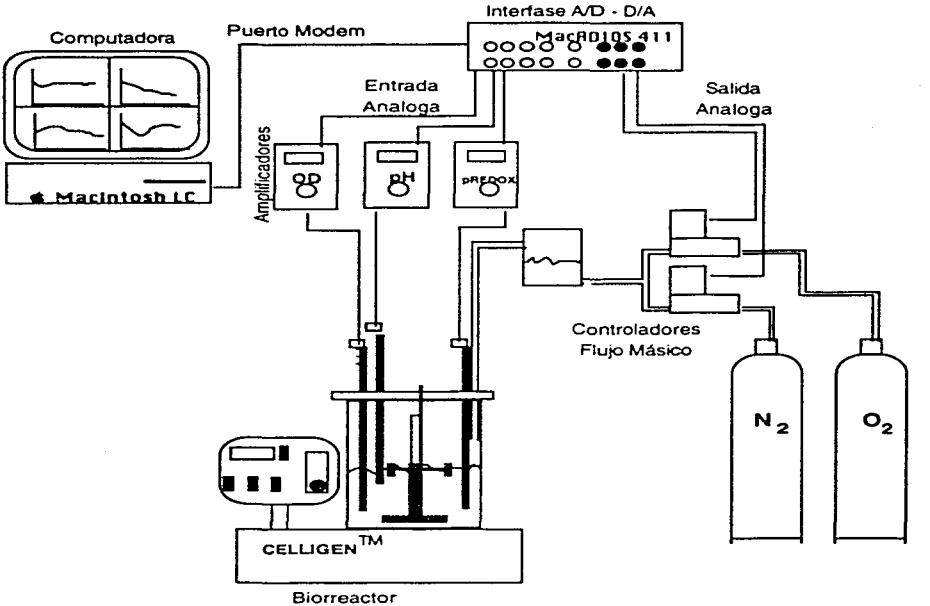

Figura IV.1 Sistema de control y adquisición de datos.

IV.6 Estudios en flujos laminares y turbulentos.

Muestras de frascos agitados en fase exponencial fueron diluídas con medio de cultivo a una densidad celular de ca. 1.0 x 10^6 cel/mL. 15 mL fueron sometidos a una velocidad de deformación de 3,000 s^{-1} utilizando un reómetro Contraves Rheomat 120 con un dispositivo *double gap* (MS0/115). El dispositivo consiste en un cilindro fijo con 4.6 cm de diámetro, un cilindro móvil de 4.66 cm de diámetro y un vaso externo de 4.8 cm de diámetro. Esta configuración garantizó la ausencia de daño a las células en la interfase gas líquido, que tenía únicamente una relación área/volumen de 0.045 cm^{-1}. La temperatura se controló en 27°C. Además de los ensayos en el reómetro, muestras de 0.5 mL fueron sometidas a condiciones

turbulentas y a la presencia de interfases gas-líquido a través de agitarlas en un vortex (Genie 2, Fisher) a máxima velocidad.

IV.7 Métodos analíticos

IV.7.1 Determinación de concentración, viabilidad y tamaño celular.

La concentración y el tamaño celular se determinaron utilizando un contador de partículas Coulter (Coulter Multisizer II, Coulter Electronics) con un tubo de apertura de 100 µm y utilizando solución isotónica Isoton II (Coulter, cat. 7546719) para diluir las muestras. La viabilidad se determinó con la técnica de exclusión de azul de tripano en un hematocitómetro.

IV.7.2 Titulación de virus

Los títulos virales se determinaron a través de ensayos en placa. Primeramente, se realizaron diluciones seriadas del lote de virus hasta 10^{-8}. En placas de cultivo de 24 pozos se colocaron $2x10^5$ cel/pozo en 450 µL de medio TNM-FH 10% SFB sin PF68. Después de que las células se adhirieron al fondo del pozo, a cada pozo se agregaron 50 µL de las diferentes diluciones de virus. Normalmente, se plaquearon las diluciones 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}. Se agitaron las placas y se dejaron reposar por 2h. A continuación se eliminó el medio de cultivo y se agregaron 500µL de agarosa de bajo punto de fusión (GIBCO, cat. 15517-022) al 1.5% p/v en TNM-FH 10%SFB. Se incubaron a 27°C. Al quinto día se agregó agarosa (GIBCO, cat. 15510-019) al 0.6 % p/v con 0.07% p/v de rojo neutro para teñir las células vivas. Después de incubar 12 h, se observan zonas donde el virus ha provocado lisis celular (placas). Las altas diluciones utilizadas hacen válida la suposición de que cada una de las placas formadas fue originada por una sola partícula viral infectiva, que después de propagarse lisó a varias células. De esta forma, los títulos virales se reportan como unidades formadoras de placa (ufp) por volumen de muestra.

IV.7.3 Determinación de glucosa, glutamina y lactato

La glucosa, glutamina y lactato se determinaron enzimáticamente con un analizador bioquímico YSI modelo 2700 (Yellow Spring Instruments). El analizador cuenta con membranas con enzimas inmovilizadas (glucosa oxidasa, glutaminasa, L-glutamato oxidasa y L-lactato oxidasa), que catalizan la reacción entre la muestra y el oxígeno para formar un derivado y peróxido de hidrógeno. La sonda del sensor contienen un sistema de electrodos amperométricos que responden ante la presencia del peróxido. El flujo de corriente en el circuito del ánodo de platino es linearmente proporcional a la concentración de glucosa, glutamina o lactato.

IV. 7.4 Determinación de fructosa

Para determinar fructosa se utilizó el "kit" No. 716260 de Boehringer Mannheim. El ensayo consiste en la fosforilación de la fructosa por la hexoquinasa. Posteriormente, la fosfoglucosa isomerasa convierte la fructosa-6-fosfato en glucosa 6 fosfato. La glucosa-6-fosfato reacciona con NADP para formar gluconato-6-fosfato y NADPH. El NADPH formado es estequiométricamente proporcional a la cantidad de fructosa. El NADPH se cuantificó por su absorbancia a 340 nm.

IV.7.5 Identificación y cuantificación de proteínas recombinantes de rotavirus

Las proteínas en muestras del paquete celular y del sobrenadante se separaron a través de geles desnaturalizantes de poliacrilamida, con un gel concentrador al 4% y un gel separador al 8 o al 12%. Las muestras se trataron como sigue: se centrifugaron 100µL de muestra a 10,000 rpm por 10 min. Las células se resuspendieron en 15 µL de agua y se agregaron 5µL de solución amortiguadora de lisis (200 mM Tris-HCl pH 6.8, 8% SDS, 0.4% azul de bromofenol, 40% glicerol, 1% 2β-mercapto etanol). 21 µL de sobrenadante se trataron con 7 µL de solución amortiguadora de lisis. Las muestras fueron hervidas 7 minutos y colocadas en el gel. Se corrieron a 150 mvolts iniciales, a corriente constante. Posteriormente, se transfirieron las proteínas a membranas de nitrocelulosa (Millipore, cat. HAHY304F0) durante 1h a 400 mvolts corriente constante, a través de un sistema semiseco (Owl Scientific, cat. HEP-1), utilizando un sistema de buffers discontinuo. Después de la transferencia, las membranas se bloquearon 30 minutos con una solución al 5% de leche descremada (Carnation) en una solución amortiguadora de fosfatos (PBS). Las proteínas recombinantes se identificaron a partir de una imunodetección en membrana de nitrocelulosa (western blot). La membrana se incubó 1h con un suero de conejo antirotavirus YM (proporcionado por los Drs. Arias y López) a una dilución de 1:2000 en PBS-0.1% leche. Después de 3 lavados con PBS-leche 0.1% (1 de 15 min. y 2 de 5 min.), la membrana se incubó 1h con un anticuerpo anti IgG de conejo conjugado con peroxidasa (Jackson Immunochemicals). Después de lavar, la membrana se reveló utilizando los reactivos quimioluminiscentes ECL (Amersham) y detectando la señal con película Kodak X-OMAT.

La cantidad relativa de las proteínas recombinantes se determinó a través de densitometría de las películas del Western blot. Las películas se escanearon y las bandas se midieron utilizando el paquete NIH Image 1.61/Fat. La intensidad y el área resultantes de la medición se multiplicaron para obtener lo que llamaremos unidades relativas densitométricas (UR). Para estandarizar entre geles, se utilizó una proteína de fusión GST-VP8, producida por *E. coli*, proporcionada por los Drs. Arias y López. La proteína de fusión se cuantificó con el método de Bradford, utilizando albúmina bovina como estándar. En la **Figura IV.2** se muestra uno de los geles utilizados para

construir la curva de calibración, y la correlación obtenida. Como se puede observar en la gráfica, esta técnica es lineal y adecuada para determinar concentraciones entre 8 µg/carril de VP8, o 60,000 UR y 1.5 µg/carril, o 10,000 UR. Para cuantificar las diferentes proteínas de rotavirus, se utilizaron unidades relativas (en adelante abreviadas como U, para distinguirlas de las unidades densitométricas, UR), donde 1 U = 1µg de VP8-GST.

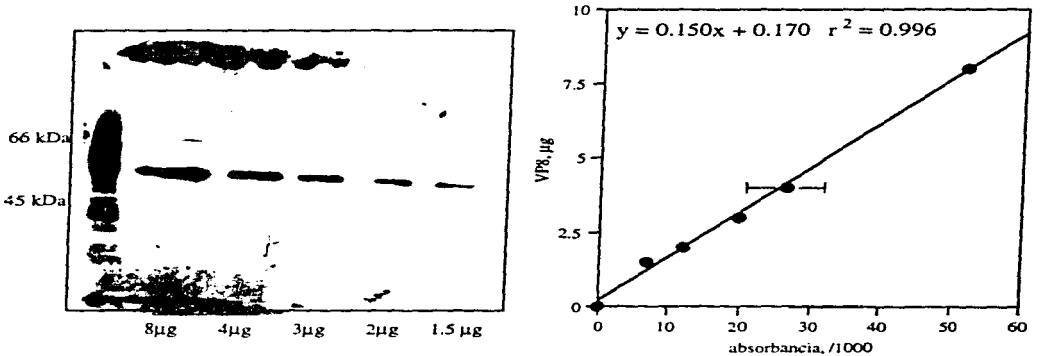

Figura IV.2 Curva estándar para cuantificación de proteínas recombinantes. Western blot de un gel de poliacrilamida al 8%. Carriles: 1, Marcador de peso molecular. 2-6, Diferentes concentraciones de GST-VP8. Se muestra media y variación entre dos curvas. En algunos puntos la desviación es menor que el símbolo.

IV.7.6 Purificación e identificación de multímeros

Muestras de diferentes volúmenes (ver **Resultados y Discusión**) de los diferentes cultivos se centrifugaron durante 10 min a 1000 rpm (**Figura IV.3**). El paquete celular se lavó 3 veces con solución amortiguadora TNC (1.58 g/L Tris.HCl, 8.18 g/L NaCl, 1.47 g/L $CaCl_2$, pH 7.5). A continuación se añadió solución amortiguadora DOC (1.21 g/L Tris, 0.037g/L EDTA, 20 g/L deoxicolato de calcio). Después de sonicar, las muestras se centrifugaron a 10,000 rpm por 10 min. En el caso de muestras, 25 µL se colocaron en geles de agarosa al 0.6% en solución amortiguadora de Tris-glicina (3 g/L Tris y 14.IV g/L glicina). Las muestras se corrieron a corriente constante a 150 volts iniciales. Los geles se transfirieron a membranas de nitrocelulosa por difusión, utilizando las mismas soluciones amortiguadoras que se utilizaron para la transferencia de proteínas. La inmunodetección se realizó como se describió en la **sección IV.5.4**. Las bandas obtenidas se compararon entre si con base en su área e

intensidad, en UR (ver sección IV.7.6). Para obtener los estándares de multímeros, los sobrenadantes obtenidos después de sonicar se pusieron en solución de cloruro de cesio (0.42 g/mL en solución TNC) y se centrifugaron durante 18h a 35,000 rpm en un rotor SW 50.1 (Beckman). Se recoletaron las bandas que constituyen las PPV. La pureza e identidad de las PPV se confirmaron por inmunodetección en membranas de nitrocelulosa (ver sección IV.7.5). Las muestras de los sobrenadantes se trataron como sigue: Las PPV se concentraron por ultracentrifugación (2h, 25,000 rpm, rotor SW 28) a través de un colchón de sacarosa ultrapura (GIBCO, cat. 15503-014) al 35% p/v en solución TNC. Posteriormente se purificaron utilizando gradientes de cloruro de cesio.

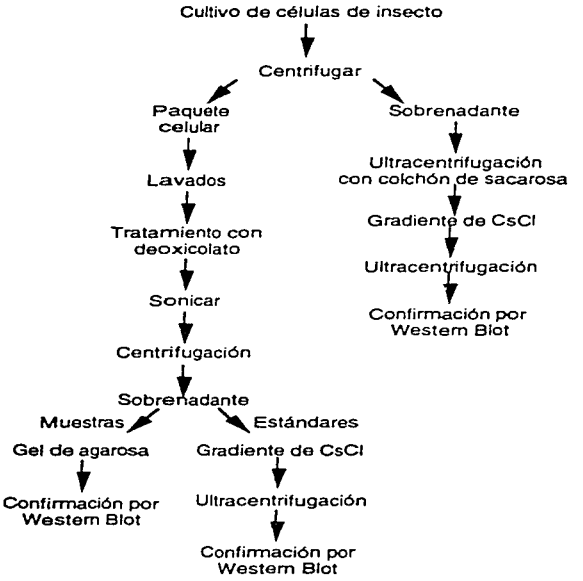

Figura IV.3 Separación de multímeros.

Capítulo V

Resultados y Discusión

V.1 Estrategia de experimentación

Como se ha discutido en los primeros capítulos, la producción adecuada de proteínas multiméricas por cultivos de células de insecto es un proceso muy complejo con varias facetas. En este trabajo se abordaron tres aspectos clave para la producción de proteínas recombinantes. Primero, el papel de aditivos protectores de los esfuerzos de corte sobre la productividad del sistema. Segundo, la producción de proteínas recombinantes en biorreactores instrumentados. Finalmente, a nivel frasco agitado, la caracterización del proceso de producción de proteínas multiméricas y el diseño de estrategias de producción. A continuación se describe brevemente la estrategia utilizada.

- Obtener información sobre los requerimientos nutrimentales y ambientales de las células, y su papel en el crecimiento celular y la producción de proteínas recombinantes.
- Desarrollar estrategias para la producción de proteínas recombinantes en biorreactores agitados. En este punto se establecieron herramientas para lograr el monitoreo y control computarizado de los cultivos y se estableció el efecto del protector de esfuerzos de corte PF68, indispensable para cultivar células de insecto en biorreactores, en la producción de proteínas recombinantes y virus. Además, se realizó el monitoreo y control computarizados en línea de los reactores con base en los resultados obtenidos en el punto anterior.
- Caracterización del proceso de producción de proteínas y de multímeros de proteínas.
- Exploración de herramientas para manipular la coexpresión de proteínas recombinantes por células de insecto.

V.2 El efecto del PF68 sobre la producción de proteínas y baculovirus recombinantes por células de insecto.

Se presenta la versión revisada del artículo "Evidence of Pluronic F-68 direct interaction with insect cells: Impact on shear protection, recombinant protein and baculovirus production", sometido a la revista *Enzyme and Microbial Technology*.

Evidence of Pluronic F-68 direct interaction with insect cells: Impact on shear protection, recombinant protein and baculovirus production

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Abstract
Pluronic F-68 has been widely used to protect animal cells from hydrodynamic stress, but its mechanism of action is still debatable. Published evidence indicates that Pluronic F-68 interacts with cells, yet, scarce information exists of its effect on recombinant protein and virus production by insect cells. In this work, the effect of Pluronic F-68 on production of recombinant baculovirus and rotavirus protein VP7 was determined. Evidence of Pluronic F-68 direct interaction with Sf-9 insect cells was also obtained. Maximum recombinant VP7 concentration and yield increased 10 times, whereas virus production decreased by 20 times, in spinner flask cultures with 0.05% (w/v) Pluronic F-68 compared to controls lacking the additive. No differences were observed in media rheology, nor kinetics of growth and infection (as inferred from cell size) between both cultures. Hence, Pluronic F-68 influenced cell physiology, independently of its shear protective effect. Cells subjected to a laminar shear rate of 3000 s-1 for 15 min, without gas/liquid interfaces, were protected by Pluronic F-68 even after its removal from culture medium. Furthermore, the protective action was immediate in vortexed cells. The results shown here indicate that Pluronic F-68 physically interacts with cells in a direct, strong, and stable mode, not only protecting them from hydrodynamic damage, but also modifying their capacity for recombinant protein and virus production.

Keywords: Insect cells, Pluronic F-68, baculovirus, recombinant protein production, shear sensitivity, protection mechanism.

Abbreviations: PF68-Pluronic F68, pfu-plaque forming units, MOI-multiplicity of infection, hpi-hours postinfection, qglc-glucose consumption rate.

Introduction

Pluronic F-68 (PF68) has been widely used as a shear protective agent for animal cells in suspension culture. Its use has resulted in increased cell survival and cell concentration, particularly in cultures with serum free media or in bioreactors where cells are subjected to deleterious shear stresses. The effectiveness of PF68 has been proven by many research groups in both, mammalian and insect cell lines (1 — 14, reviewed in 15). Nonetheless, the use of PF68 remains empirical and its mechanism of action is still a matter of debate.

Two general mechanisms of PF68 protection have been proposed, one that is physical in nature and one due to a cellular effect where an intrinsic property of the cell is influenced. The physical mechanism is supported by various experimental observations which indicate that PF68 affects the properties of the medium, reducing the level or frequency of forces experienced by the cells. For instance, it has been observed that cells are protected immediately after PF68 addition, ruling out a slower biological effect (16). Furthermore, PF68 has been shown to stabilize the foam layer and decrease the rising velocity of bubbles, thus reducing the shear forces to which cells are subjected in sparged bioreactors (10, 17, 18). These effects can be attributed to changes in culture media characteristics, such as surface tension. In addition it has been shown that PF68 can reduce cell-to-air bubble adsorption by either surrounding the bubbles or attaching to the cells, and thus avoiding cell damage upon bubble rupture (11, 13, 18). Evidence of a cellular or biological protective mechanism has also been presented indicating that PF68 can affect intrinsic properties of the cells, rendering them more tolerant to hydrodynamic damage. Namely, Ramírez and Mutharasan (6) observed a reduction of plasma membrane fluidity by PF68, which was shown to correlate with a decrease in shear sensitivity. Likewise, Zhang et al. (9) observed an increased mechanical resistance of individual cells upon addition of PF68. Interestingly, PF68 can also affect cellular metabolism, growth, and viability of mammalian and insect cells, even when they are not subjected to shear stress (1, 4, 12, 19, 20).

Some of the described protection mechanisms of PF68 could potentially have an effect on viral adhesion, infection or recombinant protein production by the insect cell-baculovirus expression vector system. For instance, the proposed PF68-cell interactions might constitute a physical barrier that could impede virus to cell adhesion. Moreover, the reported influence of PF68 on plasma membrane, metabolism and viability could affect virus internalization/budding and protein or DNA synthesis (20 — 23). Also, the virus-cell interaction could be affected by changes in physical properties of culture medium by PF68, as the main mechanism ruling insect cell-baculovirus contact is Brownian diffusion of virus in culture media (12). Nevertheless, only scarce information is available on the effect of PF68 on infection, recombinant protein and virus production by insect cells. In the present study, evidence of PF68 physical interaction with insect cells was obtained, and the effect of such interaction on cell growth, protein and virus production is discussed. The results shown in this article should be useful to further understand the mechanism of PF68 protection, and to determine if PF68 should be generally used in insect cell cultures, as it now occurs.

Materials and Methods

Cell line, culture medium and viral stock

The Sf9 cell line (ATCC CRL-1711), at an unknown passage number, was cultured at pH 6.2 and 27 °C in 100 mL spinner flasks (6.5-cm diameter) with a working volume of 60 mL and agitated at 100 rpm with a suspended magnetic bar (4.5-cm diameter). Spinners were inoculated with exponentially growing cells that had been previously subcultured at least three times with or without PF68, as noted. TNM-FH medium (prepared from Grace's medium and 3.33 g L-1 of lactoalbumin and yeastolate ultrafiltrate, Life Technologies) was used in all experiments. Medium was also supplemented, except where otherwise indicated, with 10% (v/v) fetal bovine serum (Life Technologies), and 0.05% (w/v) autoclaved PF68 (BASF). Cultures were infected at a viable cell concentration of ca. 0.3 x 106 cell mL-1 to avoid nutrient or oxygen limitation. Recombinant baculovirus producing (under the polyhedrin promoter) the VP7 protein from rotavirus strain SA11 was from Dr. Estes, Baylor College of Medicine (obtained from Dr. S. López) (24). The same viral stock for each protein was used in all experiments. The stock was maintained at 4°C until needed.

Rheometer

Samples from spinner flask cultures in exponential growth phase were diluted in fresh culture media to give a final viable cell concentration of ca. 1.0 x 106 cell mL-1. 15-mL samples were then subjected to a constant laminar shear rate of 3000 s-1 using a Contraves Rheomat 120 Rheometer with a double gap measuring system (MS0/115) (kindly lend by Dr. E. Galindo). Such a system consisted of a rotating 4.66-cm diameter middle bob, a fixed 4.6-cm diameter cylinder and a 4.8-cm diameter outer cup, which resulted in a negligible surface area to volume ratio of 0.045 cm-1. This arrangement guaranteed the absence of possible cell damage due to interaction with liquid/gas interfaces. Temperature was controlled at 27 °C, and shear stress and viscosity were continuously displayed.

In addition to tests in rheometer, 0.5 mL samples were subjected to turbulent conditions and presence of liquid/gas interfaces by vortexing at maximum speed (Genie 2, Fisher).

Analytical methods

Cell concentration and size were determined using a Coulter Multisizer II (Coulter Electronics) and cell viability by Trypan blue exclusion. Viral titers were determined in 24-well plates by plaque assays performed in triplicate as described elsewhere (25). Glucose and lactic acid were determined enzymatically with a YSI analyzer model 2700 (Yellow Springs

Instruments). Recombinant protein was detected by Western blot analysis as follows. Cell pellets or supernatants were boiled 7 min in a reducing buffer (200mM Tris-HCl pH 6.8, 8% SDS, 0.4% bromophenol blue, 40% glycerol and 1% 2-b-mercaptoethanol) and loaded in 12% SDS-polyacrylamide gels. Following electrophoresis, proteins were transferred onto nitrocellulose membranes (Millipore). After blocking the membranes with 5% non-fat dried milk, proteins were detected with a rabbit antiserum to porcine rotavirus strain YM provided by S. López, Instituto de Biotecnología-UNAM. Blots were developed after incubation with a peroxidase labeled goat anti-rabbit antibody using ECL blotting detection reagents (Amersham Life Science). Rotavirus recombinant proteins were then quantified by densitometry against a standard curve of purified GST fusion VP8 of rotavirus. As the antibody used has a different affinity for non-fused rotavirus recombinant proteins, only a relative determination of VP7 is possible. Accordingly, protein concentrations are reported as relative units (RU), where a relative unit corresponds to the equivalent of one μg of VP8-GST. A similar relative quantification approach has been used by other research groups for other recombinant rotavirus proteins (26).

Experimental approach and statistical treatment of data.

To determine if PF68 has an effect on recombinant virus and protein production by insect cells, 8 cultures, 4 with and 4 without PF68, were infected with the baculovirus encoding VP7 with a MOI of 1 pfu cell-1. Control uninfected cultures were performed in parallel. All cultures were simultaneously sampled. Mean and standard deviations between cultures at each sample time were calculated, and are shown in Figures 1-4 and Table 1. A two-tailed t test was performed to determine if the means, shown in Table 1, were significantly different within 99% confidence limit.

Results and discussion

Effect of PF68 on growth, infection, protein and virus production kinetics

In Figure 1, sample mean and standard deviation for the cultures with or without PF68 are shown. Mean and standard deviation for kinetic and stoichiometric parameters are summarized in Table 1. Viability remained fairly constant for both type of cultures during the first 48 hours postinfection (hpi) and later decreased (Fig. 1A). Cultures were harvested when viability decreased below 25%. As expected with the MOI used, total and viable cell concentration increased ca. 2 times after infection in medium with or without PF68. Viable cell concentration increased during the first 48 hpi, and then continuously decreased in both cultures

(Fig. 1B). A comparable behavior has also been observed by others in cultures infected at similar cell concentrations and with the same MOI (27). A low cell density at infection was chosen to assure nutrient and oxygen excess at all times, which was confirmed by the absence of lactate production and non-limiting glucose concentration (28), as shown in Figure 1C. Initial lactate concentration originated from serum supplements. No significant difference was observed between maximum specific glucose consumption (q_{glc}) and cell yields on glucose for both conditions tested (Table 1). In non-infected cultures maintained at the same conditions, similar growth kinetics were observed with or without PF68 (data not shown). Accordingly, for the culture conditions used in this study, there is no evidence of a deleterious hydrodynamic effect.

Recombinant protein production began 24 hpi and accumulated until harvest for cultures with PF68 (Figure 2). In cultures without PF68, recombinant protein concentration accumulated until 71 hpi, and remained constant between 71 and 91 hpi. Thereafter, total recombinant protein concentration remained constant but subsequently decreased due to degradation. Even when growth kinetics in cultures with or without PF68 were similar, protein production was increased 10 times by PF68 addition. In Table 1, it can be observed that the maximum recombinant protein concentration, yield, productivity, and specific production rate were 10 times higher in cultures with PF68 compared with cultures lacking the additive. The observed differences were determined to be significant with 99% confidence limit. These results clearly indicate that PF68 has a biological or metabolic effect, independent of its shear protective characteristics.

An important difference between intracellular and secreted VP7 concentration was also observed between cultures with and without PF68 (Figure 2, panels B and C). It can be seen that for cultures with PF68, extracellular VP7 concentration increased 12.4-fold with respect to cultures without PF68, whereas its intracellular concentration was only tripled. VP7 is a 38 kDa glycosylated protein, 5% of its weight corresponding to the carbohydrate residues (29). No detectable difference was observed between the molecular weight of intracellular or extracellular VP7 in Western blots from 12% polyacrylamide gels. Therefore, the behavior observed in Fig. 2 could not be attributed to differences in the ability to perform postranslational modifications by cells cultured with or without PF68. Another explanation would be that presence of PF68 increases extracellular transport of the recombinant protein, since changes in cellular transport have been reported as a consequence of modified plasma membrane fluidity or PF68 addition (22, 30). Protein stabilization or inhibition of degradation by PF68 could also be responsible for the results obtained (31). Nevertheless, all these hypotheses remain to be proven.

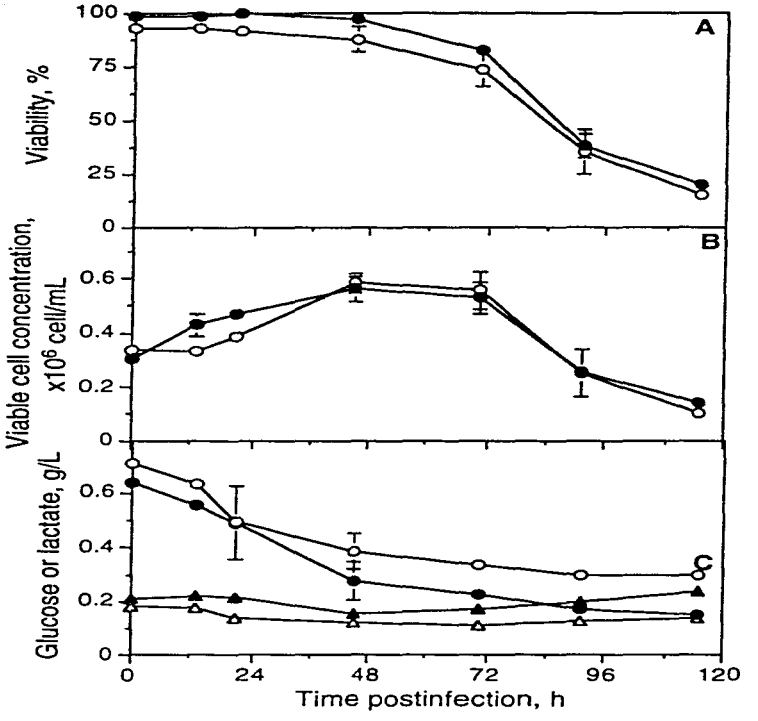

Fig. 1. Typical behavior of infected cultures with and without PF68. Each symbol represents the mean of 4 simultaneous experiments. The bars represent the standard deviation between such experiments. In some cases, the bars are smaller than the symbols. A; Viability. B; Viable cell concentration. C; Glucose , ●. and lactate Δ. Closed symbols with 0.05% PF68, open symbols without PF68.

Table 1. Virus and recombinant protein production with and without 0.05% PF68.

	Without PF68	With PF68
Maximum q_{glc} x10^{12} (g cell^{-1} h^{-1})	16.77 ± 1.1	17.84 ± 2.66
Overall yield x10^{-9} (cell g_{glc}^{-1})	0.72 ± 0.01	0.70 ± 0
*Max. VP7 conc. (RU mL^{-1})	242.36 ± 75	2451.45 ± 600
*Yield x10^{6} (RU VP7 cell^{-1})	409 ± 104.1	4312.45 ± 706
*Productivity (RU VP7 mL^{-1} h^{-1})	5.39 ± 1.66	54.48 ± 13.33
*q_{VP7} x10^{6} (RU cell^{-1} h^{-1})	9.01 ± 2.31	95.83 ± 15.62
*Max. viral titer x10^{-6} (pfu mL^{-1})	3600 ± 750	175 ± 75

Mean and standard deviation from 4 simultaneous experiments
RU= relative units (see Materials and Methods)
* Refers to parameter where mean between cultures with and without PF68 are different with a 99% confidence, as determined from a two tailed t test analysis.

As shown in Figure 3 and Table 1, virus production was over 20 times higher in cultures without PF68 than in those supplemented with the additive. Several hypotheses can be proposed to explain this behavior although further experimental evidence is needed. PF68 probably affects virus assembly, or virus budding. Interestingly, Al-Rubeai et al. (20) have observed in hybridoma cultures a reduction in DNA synthesis upon PF68 addition, but a substantial increase (70%) in monoclonal antibody titers. They did not explain nor hypothesized about the causes of such effects, but their results could explain ours. Namely, PF68 could have caused an increase in protein production and a decrease in DNA synthesis, as inferred from the virus titer.

The observed differences between cultures with and without PF68 could be due to an alteration of the infection process, probably caused by PF68 interaction with the plasma membrane. To test this, variations in cell size distributions were compared and are shown in Figure 4. An indication of an efficient infection is the increase of cell size after virus addition, as has been previously reported by Jain et al. (32) and Taticek and Shuler (33). Then, if the observed behavior was in fact caused by a modification of the infection process, a difference between cell size distribution of populations with or without the additive would be expected. However, this was not the case. Mean cell diameter increased ca. 17% after infection for all cultures, with a similar distribution throughout the entire cultures. Thus, PF68 did not affect the rate of initial cellular infection. A similar conclusion was previously reported by Kioukia et al.

(12) from microscopic observations of cultures infected with wild-type baculovirus at a MOI of 10 pfu cell-1.

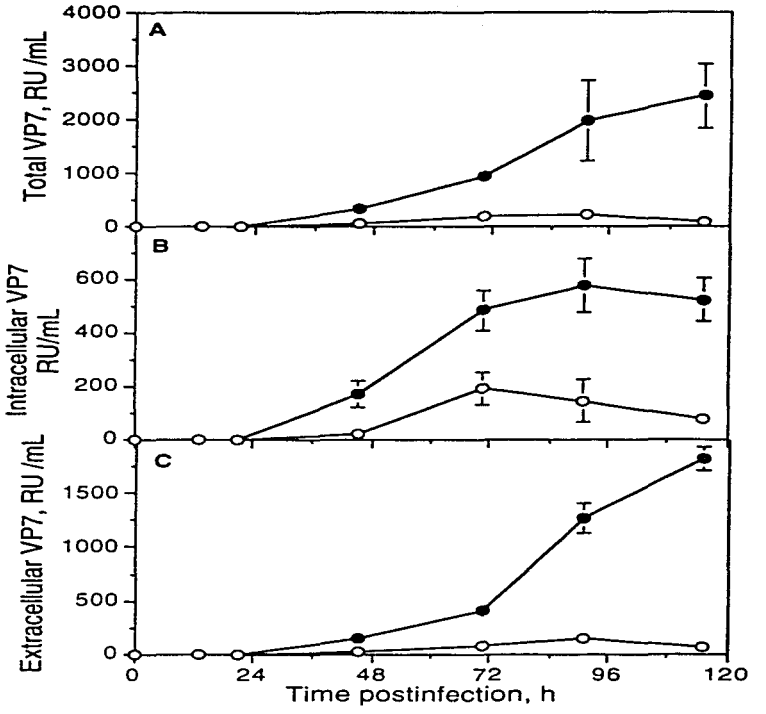

Fig. 2. Recombinant VP7 production with and without PF68. Each symbol represents the mean of 4 simultaneous experiments. The bars represent the standard deviation between experiments. In some cases, the bars are smaller than the symbols. A, Total; B, Intracellular; C, Extracellular recombinant protein concentration. Closed symbols with 0.05% PF68, open symbols without PF68. RU refers to relative units as detailed in the Materials and Methods section.

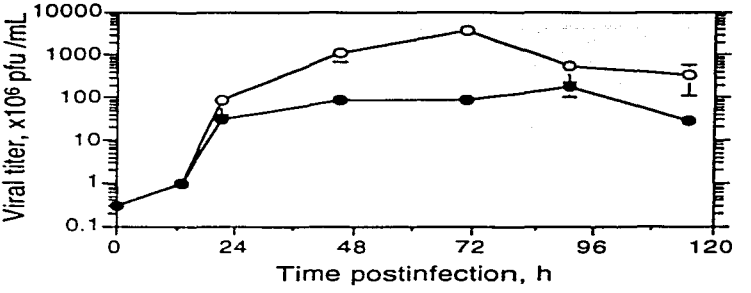

Fig. 3. Viral titer kinetics with ● 0.05% and without ○ PF68. Each symbol represents the mean of 4 simultaneous experiments. The bars represent the standard deviation between such experiments. In some cases, the bars are smaller than the symbols.

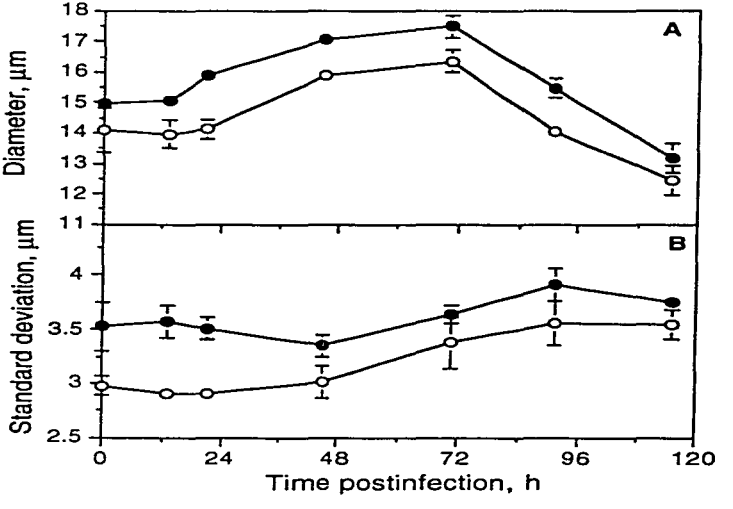

Fig. 4. Mean cell diameter and standard deviation after infection. A, Diameter; B, Standard deviation. Closed symbols with 0.05% PF68, open symbols without PF68. Each symbol represents the mean of 4 simultaneous experiments. The bars represent the standard deviation between such experiments. In some cases, the bars are smaller than the symbols.

However, no information regarding the effect of PF68 on secondary infection was obtained by them, as 100% of the population was initially infected due to the high MOI used. Assuming that infection of cultures shown in Figure 4 followed Poisson distribution (34 — 36), only 37% of the cell population should be initially infected. As a decrease of viral progeny was observed in cultures supplemented with PF68, a reduced secondary infection could be expected. Nevertheless, even when PF68 addition reduced viral titers almost 20 times, the produced viral progeny (30 x 10^6 pfu $cell^{-1}$) was more than enough to infect the remaining 63% of the cellular population not initially infected. This can be seen in Figure 4 as a further increase in mean cell diameter from 24 to 48 hpi in both cultures. Thus, PF68 did not affect secondary infection. Similar results (not shown) as those reported in Table 1 and Figures 1-4 were obtained in experiments performed in duplicate using a viral stock with a different passage number.

Evidence of biological shear protective mechanism by PF68 in insect cells from viscometric studies

Further experiments were conducted to understand the mechanism of PF68 action, specifically, to investigate if it interacts directly with insect cells, as has been reported for mammalian cells (6, 9). Approx. 1 x 10^6 cell mL^{-1} were subjected to a laminar shear rate of 3000 s^{-1}, as described in the Materials and Methods section. In agreement with Goldblum et al. (5), no difference was observed between viscosity of medium with 0.05% w/v or without PF68. As can be seen in Figure 5A, cell viability without PF68 declined after exposure of only 3 min to a shear stress of 4.51 N m^{-2}, while an exposure of 15 min decreased viability below 32%. PF68 protected the cells even after being subjected to the same shear stress for 15 min. Murhammer and Gooche (2), have previously suggested that the Trypan blue uptake method can overestimate viability due to artifacts caused by a possible direct interaction of PF68 with the plasma membrane. Since we measured viability by Trypan blue exclusion, such adduced artifact was discarded by confirming that same low viabilities were obtained with and without PF68 in severely stressed cultures never grown previously with the additive. The critical shear stress for Sf9 insect cells in medium without additives, and absence of gas/liquid interfaces, has been reported to be 0.59 N m^{-2} (5), in agreement with our results. Therefore, cellular resistance to a shear stress of 4.51 N m^{-2} for 15 min was considered as an indication of PF68 protective action.

Evidence of a physical protection mechanism of PF68 is mainly based on studies were cells are exposed to turbulent hydrodynamic shear stresses and to the interaction of bubbles and gas/liquid interfaces (15, 37 — 39). In contrast, during viscometric studies cells are only exposed to laminar shear stresses and interaction with bubbles and gas/liquid interfaces are absent. Thus, since PF68 does not alter the rheological properties of the medium, results of the

viscometric studies can be interpreted as an indication of a direct PF68-cell interaction. Furthermore, if a direct, strong, and stable interaction of PF68 with the cells, for instance intercalation with the plasma membrane (6), is responsible for the shear protecting effect of the additive, then its action should remain after washing the cells. Thus, to test this, cells grown with 0.05% w/v PF68 were centrifuged and resuspended in PF68-free medium. Cells were then subjected for 15 min to a shear rate of 3000 s^{-1}; immediately, 30, 115 and 130 min after PF68 removal (Figure 5B). As it can be seen, protection to shear damage was observed immediately after washing the cells and remained, at least, for the following 130 min. Cells were protected from shear even 24 hours after removal of PF68, but data are not shown in Figure 5B as other changes, such as cell age and metabolism, could be also influencing the results at longer time periods. Fresh media addition was not responsible for the observed shear protection, as the same viability was observed in shear exposed cultures never grown in PF68, before and after cell washing. These results also indicate that caution should be taken when interpreting data of PF68 action from cells previously grown in presence of the additive.

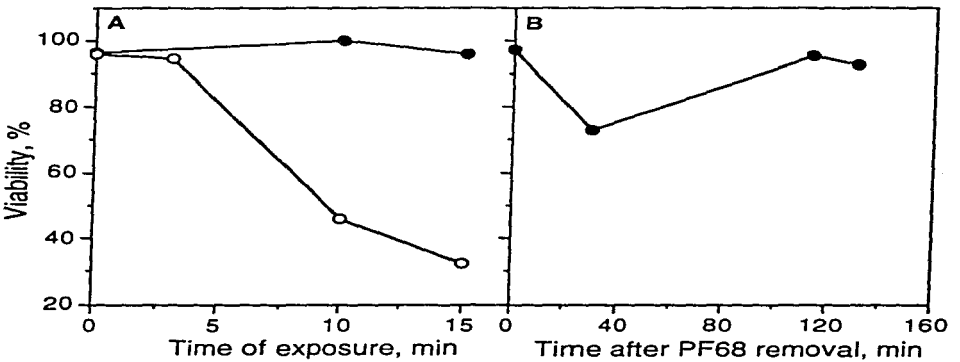

Fig. 5. Viability of cells after exposure to 3000 s-1 in a Contraves rheometer. Viability was calculated by dividing the number of viable cells after exposure by the total cell concentration before exposure. A; media ● with 0.05% and without O PF68. B; cells were exposed 15 min to 3000 s-1 after the time indicated following removal of PF68.

Wu et al. (13) reported that PF68 adsorbed to insect cells, and attributed its protective effect to the reduction of cellular hydrophobicity by additive-cell interaction. The reduced

hydrophobicity in turn, was claimed to reduce cell-bubble attachment, and therefore protected cells from bubble rupturing. In this work, we also obtained evidence of cell-PF68 interaction, but as cells were not exposed to bubbles, the explanation of Wu et al. (13) of the protection mechanism of PF68 cannot be extended to our results. Therefore, an effect of PF68 on the cell's intrinsic properties appears as the most probable explanation for the observed behavior. Such effects, for instance, can be caused by the direct interaction of PF68 with the plasma membrane or indirectly by modifying its lipid composition. Both effects can reduce the plasma membrane fluidity, and therefore, increase cellular resistance to shear. Namely, Ramírez and Mutharasan (6) have shown that PF68 can decrease plasma membrane fluidity, as measured by an increase in more than 0.01 units of fluorescence anisotropy (rs), and that cell resistance to shear damage is drastically increased even with an increase in rs of only 0.01 units. If plasma membrane fluidity of insect cells is reduced by PF68, as it occurs in mammalian cells, a wide variety of physiological properties would also be modified. Among these, plasma membrane fluidity has been reported to affect carrier mediated transport processes, respiratory electron transport, enzymatic activity, growth stimulation, differentiation, protein synthesis, and response to extracellular signals (21 — 23). All these effects could influence protein and viral productivity and, thus, explain the results shown before. Still, further work is needed to directly show that PF68 affects insect cell plasma membrane fluidity similarly to mammalian cells.

Evidence of biological shear protective mechanism by PF68 from insect cells subjected to turbulent hydrodynamic shear stress

The results with PF68, discussed above, were based on cells that had been subcultured for at least three times in presence of the additive. Thus, the postulated biological shear protection action could originate from either a direct effect of PF68 on the cells or alternatively by a PF68-mediated process, for instance changes in membrane lipid composition (22), due to the prolonged exposure to the additive. Both possibilities can result in modification of an intrinsic cellular property. To test these alternatives, cells never grown with PF68 and having an initial viability of 98% were vortexed for 30 s immediately after PF68 addition (Figure 6, panel A). Cells were protected from vortexing even by 0.05% w/v PF68, as compared to the control, and were fully protected with 0.1% w/v PF68. Moreover, the protection was immediate. This agrees with Michaels et al. (16), who found that PF68 protection to mammalian cells is immediate if its concentration is over 0.1% w/v. In general, metabolic responses and changes in membrane lipid composition are much slower than the time taken to perform the experiments shown in Figure 6A (28, 40 — 42). Accordingly, modification of cellular intrinsic properties

by direct physical PF68–cell interaction, rather than by a PF68-mediated process, appears to be a more reasonable explanation of the observed behavior.

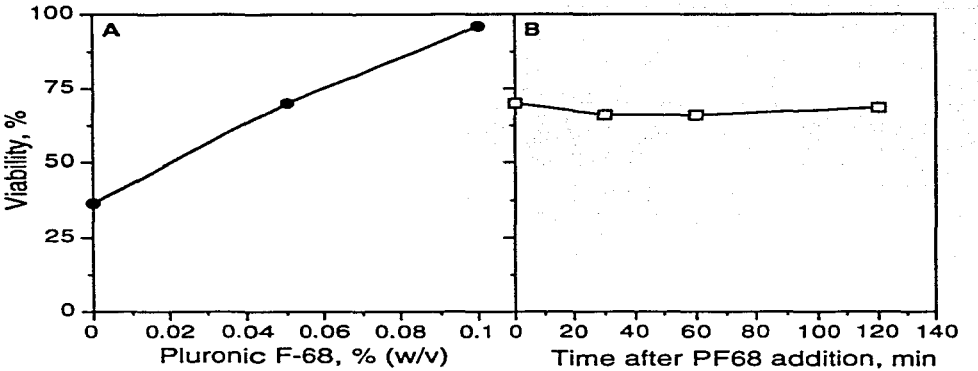

Fig. 6. Viability of cells after 30 s exposure to a vortex at maximum speed. A; the indicated PF68 concentration was added to cells immediately prior to vortexing. B; cells were supplemented with 0.05% PF68 and then vortexed after the indicated time.

Finally, to further support the hypothesis of a direct cell-PF68 interaction responsible for the shear protective effect, cells supplemented with 0.05% w/v of PF68 were vortexed after 30, 60 and 120 min of PF68 addition. As shown in Figure 6B, no difference was observed on cell survival with increased time of exposure to the additive. Exposure of cells to the additive for 18 h (data not shown) did not increase protection to shear. Accordingly, the hypothesis that the observed shear protection is related to a slower process mediated indirectly by PF68, such as changes in metabolism or membrane lipid composition, can be discarded.

Conclusions

PF68 has been extensively added to insect cell cultures as a shear protective agent, but without further evaluation of its effect on recombinant baculovirus and protein production. In this work, we have shown that PF68 has a biological impact that goes beyond its shear protective effect. Furthermore, we found that a direct physical interaction of the additive with

insect cells exists. PF68 increased recombinant protein production, even though maximum viral titers were reduced 20 times. The observed effect was not caused by differences in infection or growth kinetics of cells grown with or without the additive, nor by differences in the rheological properties of culture medium.

Recombinant baculovirus is produced during the late protein production phase of the virus life cycle, whereas recombinant proteins under the polyhedrin promoter are synthesized until the very late protein production phase. Therefore, variations in the protein/baculovirus production ratios by PF68 indicate that this medium supplement could be interfering or regulating the transition between protein expression phases. Specifically, the late protein production phase appears to be shortened with PF68 addition, while the very late protein production phase appears to be prolonged. Over the last few years, evidence has accumulated showing that PF68 affects cellular characteristics, including changes in plasma membrane fluidity, mechanical resistance, and metabolism, even under mild hydrodynamic conditions. Such effects could, in principle, affect protein synthesis and gene regulation, and thus, explain the results obtained here.

The use of viscometric studies in the absence of gas-liquid interfaces, provided further evidence that PF68 protection mechanism is not only physical and not only related to its property of separating cells from deleterious bubbles. Moreover, the observed prolonged effect of PF68 on cell resistance to shear, even after its removal from culture medium, and its fast protective action, indicate the existence of a stable and direct interaction between PF68 and the cells. Such an interaction could be causing the observed important physiological differences with respect to cultures without the additive. Furthermore, these results alert that caution should be taken when assessing the effect of the additive on cells previously grown with PF68. Finally, the results shown in this paper indicate that PF68 should be reduced when virus production is intended, whereas it should be added to increase protein productivity.

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V.3 Artículo

An insight into insect cell metabolism through selective nutrient manipulation

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An insight into insect cell metabolism through selective nutrient manipulation

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Abstract

Nutritional information of insect cell cultures, required for designing strategies to increase protein productivity, is still limited. In this work, nutrient feeding or removal was used to determine the role of selected nutrients and their interactions in insect cell metabolism before viral infection. Glucose, glutamine, tyrosine, and methionine were consumed at the highest rates in batch and fed-batch cultures, and thus were selected to be manipulated. Glucose and glutamine could be replaced by other carbon sources or amino acids, respectively. However, growth was reduced when such alternative sources were utilized, revealing the distinctive role of glucose and glutamine. Glucose and glutamine consumption rates were regulated by their own concentration, although glutamine concentration did not affect glucose consumption rate or vice versa. Excessive glucose or glutamine supply caused energy and carbon wastes, as observed by reduced cell yields and accumulation of by-products, such as alanine. Nevertheless, growth inhibition was attenuated by simultaneous excess of both nutrients, probably because cells had both carbon and ammonia available to deal with toxic by-products in the form of alanine. Other amino acids, such as tyrosine and methionine, were also relevant for maintaining prolonged viability. The importance of an adequate nutrient supply was demonstrated; particularly, the need to maintain glucose and glutamine concentrations just above their critical values. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Insect cells; Metabolism; Nutrients; Amino acids; Carbohydrates; Glucose; Glutamine; Sf-9

1. Introduction

The insect cell-baculovirus expression vector system has become widely used for academic purposes and is being considered for production of pharmaceutical proteins, some already on clinical trials (Yang et al., 1996). Successful exploitation

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of this technology at a commercial scale will depend on attaining high viable cell concentration cultures, which in turn can yield highly productive processes. This can be accomplished if suitable culture conditions are maintained before infection, including availability of nutrients and reduction of toxic or futile by-products. However, information on nutritional requirements of insect cell cultures is still incomplete. Such information is needed for maintaining optimum culture conditions, and designing selective nutrient feeding strategies, aimed at lowering culture costs and enhancing productivity.

As for other animal cells, glucose and glutamine have been generally considered as the key nutrients for insect cells (Grace and Brzostowski, 1966; Mitsuhashi, 1982, 1989; Roberts, 1984; Kamen et al., 1991; Stavroulakis et al., 1991; Reuveny et al., 1992; Bedard et al., 1993; Ferrance et al., 1993; Wang et al., 1993a,b). The exhaustion of glucose, or other monosaccharides in culture media, has been reported to induce cessation of exponential cell growth (Bedard et al., 1993; Drews et al., 1995; Palomares and Ramirez, 1996). Likewise, glutamine has been considered as an essential nutrient for insect cells (Mitsuhashi, 1982; Roberts, 1984; Mitsuhashi, 1989; Stavroulakis et al., 1991; Ferrance et al., 1993), although it has been recently reported that Sf-9 cells can synthesize it if adequate culture conditions are maintained (Öhman et al., 1996). Nonetheless, glucose and or glutamine feeding has failed to significantly increase cell concentration or protein productivity (Roberts, 1984; Caron et al., 1990; Ferrance et al., 1993; Nguyen et al., 1993; Wang et al., 1993a; Lee and Park, 1994), indicating that other unidentified factors are limiting cell growth. Some groups have attempted to avoid nutrient limitation by feeding complex undefined supplements, or by replacing spent medium with fresh medium. These strategies have resulted in increased cell and protein concentration (Roberts, 1984; Lazarte et al., 1992; Lindsay and Betenbaugh, 1992; Nguyen et al., 1993; Bedard et al., 1994; Hensler and Agathos, 1994; Lee and Park, 1994; Kioukia et al., 1995; Tom et al., 1995). Some of the nutrients that have been added with good results are yeastolate, yeastolate and amino acids, and serum (Roberts, 1984; Reuveny et al., 1993; Bedard et al., 1994; Lee and Park, 1994; Drews et al., 1995). Still, the effect of a particular nutrient on cell physiology and a full identification of the specific components that limit growth or cause cell death can hardly be established through such approaches.

Efforts have focused on determining the requirements of insect cells and designing culture media through qualitative observations: either cells grow or not (Grace and Brzostowski, 1966; Clements and Grace, 1967; Mitsuhashi, 1982, 1989; Reuveny et al., 1992). Recent information has appeared where the nutritional needs of insect cells and the effect of feeding individual nutrients, such as glucose and glutamine, have been determined (Caron et al., 1990; Kamen et al., 1991; Nguyen et al., 1993; Wang et al., 1993b; Bedard et al., 1993, 1994; Drews et al., 1995). However, only few reports have analyzed insect cell metabolism as a whole, proposing metabolic pathways and analysing the role of each nutrient in this complex system (Tremblay et al., 1992; Bedard et al., 1993; Ferrance et al., 1993; Drews et al., 1995; Öhman et al., 1995). Further research in this direction is desirable to achieve a better understanding of insect cell metabolism. A strategy that has been proposed to define metabolic fluxes and the relation between various nutrients is to maintain one or more substrates in limiting concentrations, and analyze their effect on growth and nutrient consumption kinetics. Using this strategy, Öhman et al. (1995) have described metabolic switches induced by glucose and or glutamine concentration in insect cell cultures. Additional nutrients essential to insect cell growth, such as other amino acids, may also have an important effect on cell metabolism, but have not been studied in detail.

In this work, selective nutrient removal or addition to uninfected insect cell cultures was established as an alternative for studying specific aspects of insect cell metabolism. Glucose, glutamine, methionine and tyrosine, alone or in combination, were manipulated as these nutrients have been reported to be fundamental for insect cell growth (Mitsuhashi, 1982; Tremblay et al., 1992; Ferrance et al., 1993). Culture kinetic parameters were determined, which allowed the

assessment of the role of individual nutrients and their interactions in cell metabolism.

2. Materials and methods

2.1. Cell line and culture media

The insect cell line Sf-9 (ATCC 1711) was used in this study. All cultures were seeded at a cell concentration of 0.2×10^6 cell ml^{-1} and kept at 27°C. Cells were grown in 200 ml spinner flasks with a working volume of 50 or 60 ml, as indicated, and agitated at 100 rpm with a suspended magnetic bar impeller. TNM-FH medium (Sigma, T-1032), supplemented with 10% fetal bovine serum (Sigma, F-2449) and 1% of antibiotic–antimycotic solution (Sigma, A9909, penicillin 100 000 U l^{-1}, streptomycin 100 mg l^{-1} and amphotericin B 250 μg l^{-1}) was used in all cultures. Inocula for all experiments were centrifuged to eliminate spent medium.

2.2. Analytical methods

Samples for analysis were taken daily in all experiments. Cell number was determined in a Coulter Counter Multisizer II (Coulter Electronics). Viability was determined via trypan blue exclusion by counting 200 cells from each sample in a hematocytometer. Glucose and lactate were determined enzymatically using a YSI Biochemical Analyzer model 2700 (Yellow Spring Instruments). Sucrose was determined in the YSI analyzer, after its hydrolysis to glucose and fructose with β-fructosidase (Boehringer Mannheim, 104914). Briefly, 30 μl of sample were incubated with enzyme in excess (93 U) for 15 min at 37°C. Fructose was determined with an enzymatic kit (Boehringer Mannheim, 716260). Amino acids and ammonia were determined by ninhidrin postderivatization using a Beckman Amino Acid Analyzer model 6300, as described previously (Palomares and Ramírez, 1996).

2.3. Experimental design

To assess the role of nutrients in insect cell metabolism, four sets of experiments were conducted.

I. A batch culture with a working volume of 60 ml in fully supplemented TNM-FH medium.

II. Cultures with selective nutrient removal. Cultures in the middle of the exponential growth phase (third day) were centrifuged and resuspended in medium lacking glucose or glutamine. A culture resuspended in fresh complete medium was used as a control and a batch culture was simultaneously performed. All cultures had a working volume of 50 ml.

III. Fed-batch cultures, set A. 50 ml cultures were fed as described in Table 1 with glucose and or

Table 1
Substrate additions to fed-batch cultures

Experiment	Nutrient addition schedule				
	Glucose (0.2 mmol)	Glucose (0.1 mmol)	Glutamine (0.1 mmol)	Methionine (0.017 mmol)	Tyrosine (0.02 mmol)
Set III					
–Glucose	*	–	–	–	–
–Glutamine	–	–	*	–	–
–Glucose, glutamine	*	–	*	–	–
Set IV					
–glucose, methionine, tyrosine		*		**	**
–glucose, glutamine, methionine, tyrosine		*	*	**	**

* Nutrient added on days 4, 6, 7, 8, 10, 11 and 12 of culture.
** Nutrient added on days 6, 8 and 10 of culture.

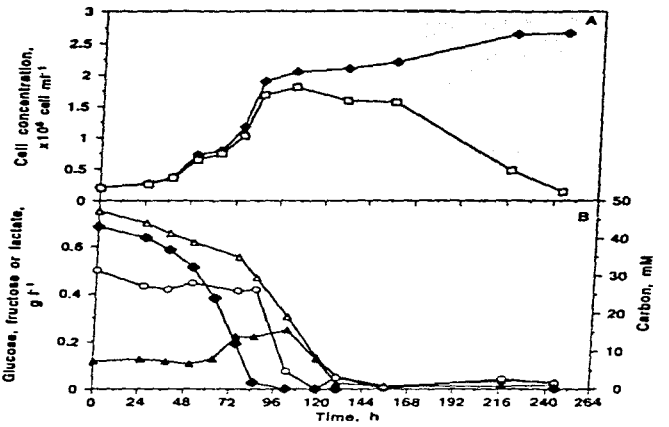

Fig. 1. Typical behavior of insect cells in batch culture. (A) Viable cell concentration (□) and total cell concentration (◆). (B) glucose (◆), fructose (□), lactate (▲) and carbon (○).

glutamine. All additions were less than 2% of the volume of the culture. The nutrients were added to raise their concentrations to their original level in TNM-FH medium, assuming that the added nutrient was depleted at the time of addition. A batch culture was performed simultaneously as a control.

IV. Fed batch cultures, set B. Two 50 ml cultures were fed with glutamine and or glucose, methionine and tyrosine, as described in Table 1, with the same considerations as in set III. A batch culture was also performed simultaneously.

2.4. Determination of specific consumption rates

The specific carbon consumption rate refers to the carbon consumed in the form of glucose, fructose and lactate, as more than one of these carbon sources were consumed simultaneously. The specific consumption rates of substrate i, q_i, were determined and calculated during the exponential growth phase after medium exchange from the following equation:

$$q_i = \left(\frac{dS_i}{dX_v}\frac{dt}{dt}\right)\mu_a$$

where S_i is the concentration of substrate i, μ_a is the apparent specific growth rate, X_v is the viable cell concentration, and t is time.

3. Results and discussion

3.1. Growth and nutrient consumption of insect cells in batch culture

Growth and nutrient consumption of Sf-9 cells in an agitated batch culture are shown in Fig. 1 and Tables 2 and 3. All other batch cultures,

Table 2
Amino acid consumption production by insect cells in batch culture

	Specific consumption rate ($\times 10^{-10}$ mmol cell^{-1} h^{-1})	% Consumed in culture
Glutamine	0.37	86.97
Cystine	0.04	100
Tyrosine	0.03	21.25
Methionine	0.02	62.41
Glutamate	−0.07*	−23.81*
Alanine	−0.23*	−42.95*

* Negative sign indicates production

performed simultaneously to other sets of experiments, had a similar behavior. Simultaneous operation of batch control cultures allowed the discrimination of experimental variation due to inoculum characteristics. Viable cell concentration increased up to 1.84×10^6 cell ml^{-1} during the first 100 h of culture, with a specific growth rate of 0.022 h^{-1}. Glucose was consumed during exponential growth phase at a rate of 0.78×10^{-10} mmol cell^{-1} h^{-1} (corresponding to 4.7×10^{-10} carbon mmol cell^{-1} h^{-1}). This rate is similar to other reports of insect cell cultures under the same conditions (Nguyen et al., 1993; Hensler and Agathos, 1994; Drews et al., 1995; Kioukia et al., 1995). Cells grew exponentially until glucose depletion, and remained in the stationary growth phase for the next 3 days. Fructose was consumed only after glucose concentration decreased below 0.02 g l^{-1}. A possible oxygen limitation could be inferred from the increase in lactate concentration after cell concentration reached 0.76×10^6 cell ml^{-1}, and while glucose and fructose were still present. It is widely recognized that lactate is produced in insect cell culture during oxygen limitation, as long as other carbon sources are available (Wang et al., 1993b; Bedard et al., 1994; Rhiel and Murhammer, 1995; Palomares and Ramirez, 1996). Lactate remained below 0.3 g l^{-1}, which was previously shown not to be toxic to insect cells (Palomares and Ramirez, 1996). Upon fructose and glucose limitation, lactate was consumed, as has been reported before (Kamen et al., 1991; Bedard et al., 1993; Palomares and Ramirez, 1996). A specific uptake rate of 4.22×10^{-10} carbon mmol cell^{-1} h^{-1} (Table 2) was calculated when two carbon sources were simulta-

Table 3
Maximum cell concentration and kinetic parameters of insect cell cultures in spinner flasks

	Maximum specific growth rate (h^{-1})	Maximum viable cell concentration ($\times 10^6$ cell ml^{-1})	Maximum carbon specific consumption rate ($\times 10^{-10}$ ml cell^{-1} h^{-1})
Set I			
Batch culture	0.022	1.84	4.7
Set II			
Batch culture	0.02	2.14	4.34
Complete replacement	0.019	3.30	4.34
Without glucose	0.015	2.64	1.50
Without glutamine	0.012	2.18	3.01
Set III			
Batch culture	0.019	1.91	4.07
Glucose added	0.02	2.25	7.02
Glutamine added	0.018	1.50	4.39
Glutamine and glucose added	0.019	2.66	6.10
Set IV			
Batch culture	0.021	2.17	N.D.
Glucose, methionine and tyrosine	0.02	2.00	N.D.
Glucose, methionine, tyrosine and glutamine	0.026	1.70	N.D.

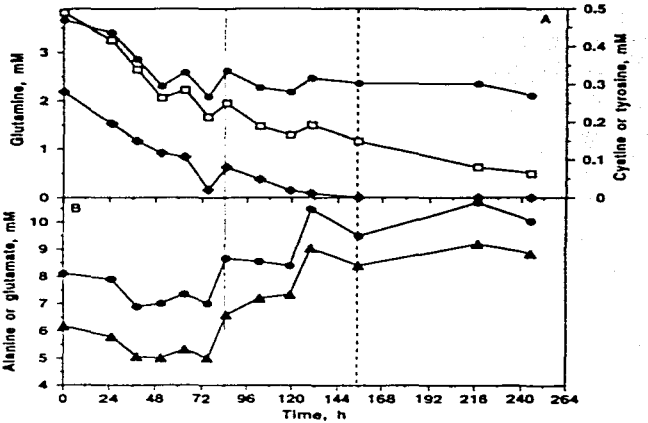

Fig. 2. Consumption or production kinetics of selected amino acids in batch culture. (A) Glutamine (□), cystine (◆) and tyrosine (●). (B) Glutamate (●) and alanine (▲). Dotted horizontal line denotes the onset of stationary phase, dashed line denotes the end of the stationary phase

neously consumed (between 85 and 130 h). This carbon uptake rate is similar to that obtained when only glucose was consumed, although the culture was no longer proliferating. Such a high carbon uptake rate of cells in the stationary phase might reflect the need to synthesize glucose de novo, to satisfy requirements that neither lactate nor fructose can fulfill, such as the pentose-phosphate pathway or for glycosilation of proteins. The other carbohydrate present in TNM-FH medium, sucrose, was not consumed (data not shown).

The amino acids with the highest consumption or production rates in batch culture are shown in Fig. 2 and Table 2. Cystine was the only amino acid completely consumed. Although cystine is an essential amino acid for Sf-9 cells (Tremblay et al., 1992), its concentration is seldom measured and its role in insect cell metabolism has not been determined. Cystine is also important for regulation of the intracellular redox potential, and its privation has been linked to triggering apoptosis in mammalian cell cultures (Mercille and Massie, 1994). In this culture, cell viability decreased after cystine concentration decreased below 0.02 mM, while cystine depletion coincided with the onset of the death phase. However, we have previously observed that even when cystine is still available, insect cell cultures enter the stationary and death phases (Palomares and Ramírez, 1996). The amino acid with the highest consumption rate was glutamine, as has also been previously observed by Wong et al. (1994). Tyrosine and methionine were also constantly consumed by the cells, although their concentration did not decrease below 0.2 mM (data not shown).

Ammonia concentration remained fairly constant at 1.2 mM until the end of the stationary

phase (data not shown). Later, its concentration increased continuously and reached 2.6 mM at the end of culture. This behavior has also been reported by several authors, and has been related to the cessation of alanine synthesis (Bédard et al., 1993; Drews et al., 1995; Öhman et al., 1995). Alanine and glutamic acid were produced from the beginning of culture and their synthesis ceased when monosaccharides were exhausted. Öhman et al. (1995) observed a decrease in alanine synthesis when cells were glucose-limited, and concluded that glucose was the main source of carbon for alanine synthesis. Thus, alanine and glutamic acid production can be a consequence of an oversupply of their precursors, pyruvate and α-ketoglutarate, which exceeds the capacity of the tricarboxylic acid cycle and the respiratory chain. It has been reported that organic acids, such as pyruvic or succinic acid, are additional by-products that can be excreted by insect cells under conditions of carbon excess (Bédard et al., 1993; Öhman et al., 1995). However, the nature of the accumulated by-product (either organic acid or amino acid) could also depend on the concentration of ammonia, as it regulates the production of alanine. Alanine synthesis has been widely recognized as a non-toxic alternative for the elimination of ammonia (Öhman et al., 1996). Accordingly, when the carbon source and ammonia are in excess, the cell will synthesize alanine and glutamate. Conversion of glucose to alanine or glutamic acid, via pyruvate and α-ketoglutarate, yields only 7% of the energy available in glucose, compared with its complete oxidation. If all the alanine and glutamate accumulated in the culture shown in Fig. 1 had originated from such a pathway, then 20% of the glucose consumed would have been wasted for alanine and glutamic acid synthesis. Moreover, alanine synthesis requires reducing power, which results in an additional energy waste by the cells (Öhman et al., 1995). Such an undesirable energy waste can be prevented by rational feeding strategies which would reduce an excessive supply of carbon sources and ammonia formation.

All other amino acids were consistently consumed in the exponential growth phase, and their concentrations remained almost unchanged during the stationary and death phases (data not shown). However, less than 20% of the initial concentration of these amino acids were consumed during all the culture. This behavior is similar to our previous report on amino acid consumption production kinetics in instrumented controlled bioreactors, although in such cultures, several amino acids were accumulated during the death phase (Palomares and Ramírez, 1996).

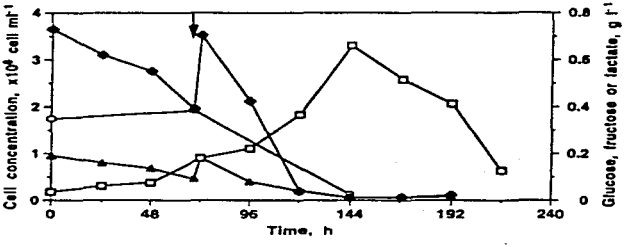

Fig. 3. Kinetics of culture with full medium exchange (control culture). Glucose (◆), fructose (○), lactate (▲) and viable cell concentration (□). Arrow indicates time of medium exchange.

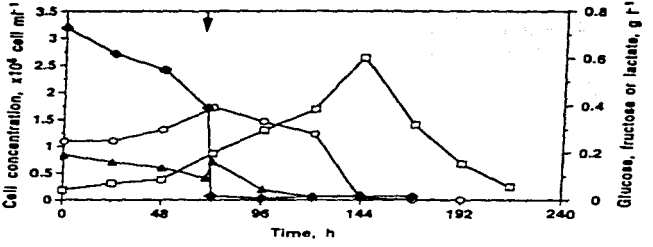

Fig. 4. Kinetics of culture with glucose removal. Viable cell concentration (□), glucose (◆), fructose (○) and lactate (▲). Arrow indicates time of medium exchange.

3.2. Culture with complete medium exchange

As shown in Fig. 3 and Table 3, the culture with complete medium replacement (control culture) reached a maximum viable cell concentration 52% higher than the batch culture performed simultaneously, while the specific growth rate and carbon uptake rate were similar. However, cell productivity (considering the total amount of medium used) of the culture with medium replacement was 10% lower than the batch culture (0.0108 versus 0.0118 × 10^6 cell ml^{-1} h^{-1}, respectively). These data indicate that medium exchange is not an effective alternative to nutrient feeding, as cost due to culture media is duplicated without a proportional increase in cell yield. It can be inferred that, at least for 50 ml cultures with cell concentrations below 3.3 × 10^6 cell ml^{-1}, dissolved oxygen was not the limiting substrate, as lactate was not produced (Wang et al., 1993b; Bédard et al., 1994; Rhiel and Murhammer, 1995; Palomares and Ramirez, 1996). It should be noted that in small volume spinner flasks, relative small changes in liquid volume can have important effects in the oxygen transfer coefficients (Aunins et al., 1989). This can explain why a higher cell concentration can be attained in the 50 ml cultures compared with the 60 ml spinners (Fig. 1), without incurring oxygen limitation.

3.3. The role of glucose

As seen in Fig. 4 and Table 3, the culture resuspended in medium without glucose reached a maximum cell concentration 80% of that obtained in the control culture. Interestingly, the culture remained in the exponential growth phase after glucose removal. This contrasts with batch cultures, which entered the stationary growth phase when glucose was depleted, even when fructose and lactate were still available. Thus, another nutrient provided by medium exchange must be limiting growth in batch cultures. The maximum specific growth rate was 25% lower in the glucose deprived culture compared with the batch and control cultures, indicating that fructose and lactate were not as effective carbon sources as glucose. Fructose and lactate consumption began immediately after medium replacement, with a specific carbon consumption rate lower than in all other cultures (Table 3). It was interesting to observe that cells have readily available all the enzymatic machinery required to shift from one carbon source to others without an adaptation phase.

Continuous feeding of glucose increased the maximum viable cell concentration by 18%, while growth rate remained unchanged (Table 3). On day 6, when glutamine and tyrosine were depleted (tyrosine data not shown), the culture entered the

stationary phase even when glucose concentration was almost 2 g l^{-1} (Fig. 5). This contrasts with the batch culture, where glucose depletion marked the onset of the stationary growth phase while glutamine and tyrosine were not exhausted (tyrosine data not shown). It has been reported that insect cells can synthesize glutamine, if ammonia is available (Öhman et al., 1995, 1996). Accordingly, glutamine synthesis was most probably occurring after its depletion, since alanine, glutamate and ammonia were consistently consumed (Fig. 5, ammonia data not shown). To further determine if tyrosine was limiting growth, Set IV of experiments was performed and results are discussed. In the glucose fed culture, the stationary phase lasted for 3 days, and the onset of the death phase did not correspond to the depletion of any nutrient measured in this work. The carbon consumption rate was almost 50% higher than in cultures where glucose was not fed, which agrees with Bedard et al. (1993), who observed a 250% increase in the specific glucose consumption rate when glucose concentration was doubled in TNM-FH medium. The high carbon consumption rate under glucose excess, and the low carbon consumption rate observed in the culture without glucose, indicates that insect cells regulate glucose metabolism similarly to mammalian cells (Miller and Blanch, 1991). This has only been scarcely reported for insect cells (Bedard et al., 1993; Öhman et al., 1995). Therefore, an optimum feeding strategy should maintain the concentration of the carbon sources just above its critical value. Despite the fact that carbon consumption rate was increased, alanine and glutamate production rates (0.12 and 0.19×10^{-10} mmol cell^{-1} h^{-1}, respectively) were similar to those observed in batch culture. This indicates that carbon excess

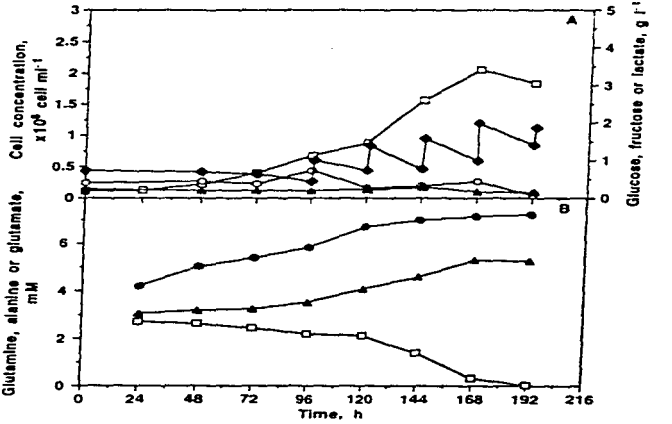

Fig. 5. Selective nutrient feeding to spinner flasks cultures. Glucose addition. (A) Viable cell concentration (□), glucose (◆), fructose (▽) and lactate (▲). (B) Glutamine (▽), glutamate (●) and alanine (▲). Data after 192 h is not shown as a power failure occurred at this time. The effect of nutrient feeding had already been established before 192 h.

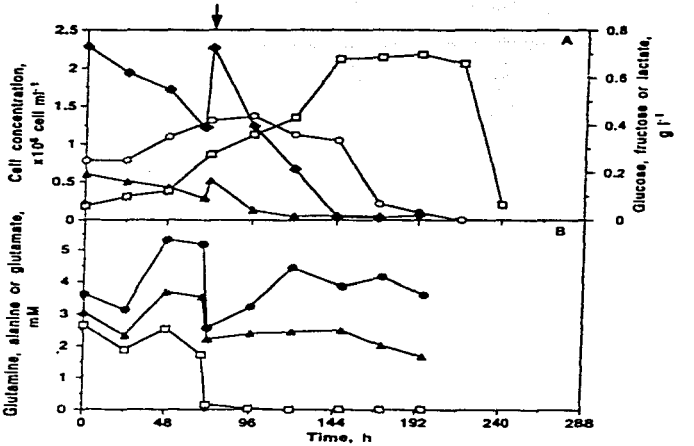

Fig. 6. Kinetics of culture with glutamine-free medium exchange. (A) Viable cell concentration (□), glucose (◆), fructose (○) and lactate (▲). (B) Glutamine (□), glutamate (●) and alanine (▲). Arrow indicates time of medium exchange.

was possibly directed to organic acid by-products as ammonia was not available for alanine and glutamate synthesis.

3.4. The role of glutamine

In the culture where glutamine was removed, a residual glutamine concentration remained (0.15 mM), probably due to carry-over from serum (Fig. 6). Nonetheless, such a residual glutamine was consumed at a much slower extent than in other cultures (0.05×10^{-10} mmol cell^{-1} h^{-1}), and was completely depleted in less than 24 h following medium exchange. Öhman et al. (1995) have also observed a reduced glutamine consumption rate in a glutamine limited culture. Cell growth continued in the absence of glutamine (Fig. 6), in agreement with previous reports (Öhman et al., 1995, 1996), but maximum specific growth rate was only 63% of the control culture. As glutamine is essential for nucleotide synthesis (Lehninger, 1982), and therefore required for cell proliferation and survival, the reduced growth rate indicates that glutamine synthesis by the cells is not as efficient as exogenous provision. During cell growth, ammonia was consumed whereas alanine was produced at a rate ca. 7 times lower than in other cultures (-0.03×10^{-10} mmol cell^{-1} h^{-1}). This indicates that glutamate is being used preferentially for glutamine production (Öhman et al., 1996), rather than for alanine synthesis. Cell growth ceased when glucose was exhausted, and the maximum cell concentration reached was only 66% of the control culture. Accordingly, cell yield on glucose was reduced by 15%, although the specific carbon consumption rate did not increase. After glucose depletion, alanine was consumed and ammonia liberated (Fig. 6, ammonia data not

shown), which indicates that pyruvate was being produced. Also, glutamate concentration decreased, suggesting that it was still being consumed for glutamine production and that its synthesis was limited by carbon availability (Fig. 6). The stationary growth phase of the culture lasted 3 days, until fructose depletion.

Glutamine feeding (Fig. 7) did not increase the specific growth rate, or the maximum cell concentration. On the contrary, the maximum cell concentration was reduced in 21%, probably because excessive glutamine supply caused the accumulation of by-products, such as ammonia, that could not be channeled through alanine synthesis. Such suspected inhibition was diminished by simultaneous glucose feeding, as discussed in the following section. The specific carbon uptake rate was similar to batch cultures, while glutamine uptake rate was two-fold higher than in batch cultures (0.8 and 0.37×10^{-10} $mmol_{Gln}$ $cell^{-1}$ h^{-1}, respectively). Thus, glutamine excess caused an increase in its uptake rate, while carbon consumption rate was not affected. The stationary growth phase initiated upon glucose depletion, and lasted until fructose was exhausted. Interestingly, the culture remained viable for a longer time period than other cultures, indicating that exogenous glutamine might be important for culture survival.

3.5. Simultaneous feeding of glucose and glutamine

Glucose and glutamine feeding prolonged the exponential growth phase for 1 day (Fig. 8). The maximum specific growth rate was similar to batch cultures, while the maximum cell concentration was 39% higher than the batch culture, 18% higher than the culture where only glucose was fed, and 73% higher than the culture where only

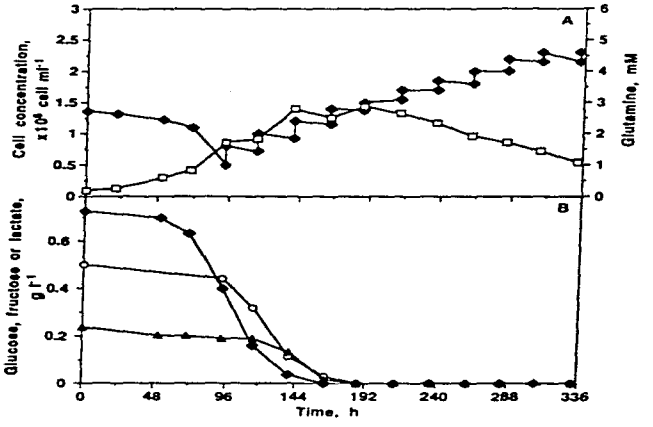

Fig. 7. Selective nutrient feeding to spinner flasks cultures. Glutamine addition. (A) Viable cell concentration (□), glutamine (◆). (B) Glucose (◆), fructose (○) and lactate (▲).

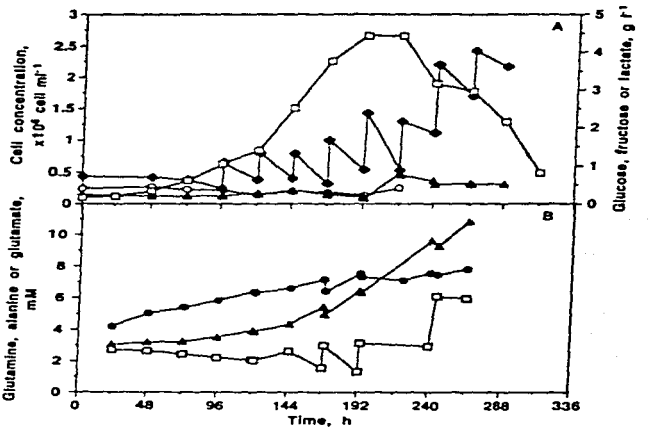

Fig. 8. Selective nutrient feeding to spinner flasks cultures. Glucose and glutamine addition. (A) Viable cell concentration (□), glucose (◆), fructose (▽) and lactate (▲). (B) Glutamine (□), glutamate (●) and alanine (▲).

glutamine was fed (Table 3). Still, a higher maximum cell concentration, compared with the corresponding batch culture, was obtained with complete medium replacement. At the end of the exponential growth phase (168 h), cystine was depleted, and the specific growth rate was reduced from 0.019 to 0.007 h^{-1}. The end of exponential growth coincided with tyrosine exhaustion, and could not be prevented by glucose and glutamine excess. The stationary growth phase lasted only 1 day.

The specific glutamine consumption rate, 0.28×10^{-10} mmol $cell^{-1}$ h^{-1}, was comparable with batch cultures, while carbon uptake rate increased in 30%, i.e. 20% less than when only glucose was fed. Alanine and glutamate specific production rates (0.27×10^{-10} and 0.14×10^{-10} mmol $cell^{-1}$ h^{-1}, respectively) were unaffected by glucose and glutamine feeding, although both amino acids accumulated during all the culture, resulting in concentrations two to three times higher than those observed in batch.

The increase in cell concentration, compared with individual nutrient feeding, indicates that simultaneous overfeeding of glucose and glutamine provided the carbon and ammonia required by the cells to deal with by-products in the form of alanine, as described previously. Various research groups have investigated the role of glutamine and glucose in insect cell metabolism. Among these, Neermann and Wagner (1996) determined that 14.25% of glutamine and 59% of glucose in Sf-21 batch cultures were completely oxidized to CO_2. Such data can be used to calculate the contribution of each nutrient to the cellular energy pool, assuming these nutrients are the only energy sources. For instance, for the culture in Set I, glucose would provide 92% of the energy, while glutamine would only contribute with 8%, as complete oxidation of glucose and glutamine

yields 32 and 27 moles of ATP, respectively. Therefore, glucose would be the main energy source, while glutamine would be mainly used for biosynthesis. This contrasts with hybridoma cultures, which use up to 55% of glutamine as an energy source (Eigenbrodt et al., 1985) and show concomitant high specific glutamine consumption rates (for instance, 4.4×10^{-10}–10^{-10} mmol cell^{-1} h^{-1}; Ozturk and Palsson, 1991).

3.6. Other amino acids

Methionine and tyrosine are essential for insect cell growth (Mitsuhashi, 1982) and, as discussed in the previous cultures, appeared to be playing a key role for cell growth. Thus, cultures were fed with these amino acids. Glucose, tyrosine and methionine feeding did not increase the cell concentration nor the specific growth rate (Table 3), although the stationary phase was prolonged for 1 day (data not shown). When glutamine was fed in addition to these nutrients, the maximum growth rate increased 30% (Table 3), and the stationary growth phase was prolonged for two more days. However, the maximum cell concentration was lower than that observed in control cultures and in the glutamine fed culture. This indicates that, although amino acid and glucose feeding failed to prolong the exponential growth phase and increase cell concentration, a supply of glucose, glutamine, methionine and tyrosine is required to maintain cultures with high viabilities for longer periods of time.

4. Conclusions

Manipulation of substrates in insect cell cultures provided interesting information about nutritional requirements, without complicated experimental strategies. In batch cultures, glucose depletion coincided with the cessation of exponential growth, even when other carbon sources, such as fructose and lactate, were present in culture media. Furthermore, when glucose was selectively removed, fructose and lactate were consumed, but only a lower growth rate than control cultures could be maintained. Thus, it can be concluded that glucose is the main carbon source for insect cell cultures and that other sources, such as fructose and lactate, are not as efficient.

Glutamine deprivation reduced cell growth rate and maximum cell concentration, indicating that, even when glutamine can be synthesized by insect cells, this is not as efficient as its exogenous provision. Glutamine consumption rate was also regulated by its own concentration, although glutamine concentration did not affect glucose consumption rate or vice versa. Such a behavior indicates that glucose and glutamine have different roles in insect cell metabolism, which cannot be fulfilled by the other. Oversupply of glutamine reduced cell concentration, unless glucose was also present in excess. This behavior is probably due to the simultaneous availability of carbon and ammonia, which allows the production of alanine, a non-toxic by-product used by the cells for ammonia and carbon surplus elimination. An excessive supply of glucose and glutamine caused an inefficient use of energy and carbon sources, evidenced by alanine production and growth inhibition. Therefore, feeding strategies should maintain both nutrients just above their critical concentrations.

Tyrosine and methionine feeding retarded cell death, even when growth rate or cell concentration did not increase. Thus, the importance of such amino acids cannot be overlooked if highly viable cultures are desired. The results of this study provide a deeper understanding of metabolism and quantitative information which can be used to design culture feeding strategies of key nutrients needed to maintain high culture viability and maximum cell concentration prior to infection.

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yields 32 and 27 moles of ATP, respectively. Therefore, glucose would be the main energy source, while glutamine would be mainly used for biosynthesis. This contrasts with hybridoma cultures, which use up to 55% of glutamine as an energy source (Eigenbrodt et al., 1985) and show concomitant high specific glutamine consumption rates (for instance, $4.4 \cdot 10^{-10}$–10^{-10} mmol cell^{-1} h^{-1}; Ozturk and Palsson, 1991).

3.6. Other amino acids

Methionine and tyrosine are essential for insect cell growth (Mitsuhashi, 1982) and, as discussed in the previous cultures, appeared to be playing a key role for cell growth. Thus, cultures were fed with these amino acids. Glucose, tyrosine and methionine feeding did not increase the cell concentration nor the specific growth rate (Table 3), although the stationary phase was prolonged for 1 day (data not shown). When glutamine was fed in addition to these nutrients, the maximum growth rate increased 30% (Table 3), and the stationary growth phase was prolonged for two more days. However, the maximum cell concentration was lower than that observed in control cultures and in the glutamine fed culture. This indicates that, although amino acid and glucose feeding failed to prolong the exponential growth phase and increase cell concentration, a supply of glucose, glutamine, methionine and tyrosine is required to maintain cultures with high viabilities for longer periods of time.

4. Conclusions

Manipulation of substrates in insect cell cultures provided interesting information about nutritional requirements, without complicated experimental strategies. In batch cultures, glucose depletion coincided with the cessation of exponential growth, even when other carbon sources, such as fructose and lactate, were present in culture media. Furthermore, when glucose was selectively removed, fructose and lactate were consumed, but only a lower growth rate than control cultures could be maintained. Thus, it can be concluded that glucose is the main carbon source for insect cell cultures and that other sources, such as fructose and lactate, are not as efficient.

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FALTAN PAGINAS.

DE 69 A LA 83

V.4 Estrategias computarizadas de control y monitoreo de reactores.

V.4.1 Artículo "The effect of dissolved oxygen tension and the utility of oxygen uptake rate in insect cell culture"

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Special Issue

The effect of dissolved oxygen tension and the utility of oxygen uptake rate in insect cell culture

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Key words: Insect cells, dissolved oxygen tension, oxygen uptake rate, metabolism, aminoacids, carbohydrates

Abstract

Dissolved oxygen tension and oxygen uptake rate are critical parameters in animal cell culture. However, only scarce information of such variables is available for insect cell culture. In this work, the effect of dissolved oxygen tension (DOT) and the utility of on-line oxygen uptake rate (OUR) measurements in monitoring *Spodoptera frugiperda* (Sf9) cultures were determined. Sf9 cells were grown at constant dissolved oxygen tensions in the range of 0 to 30%. Sf9 metabolism was affected only at DOT below 10%, as no significant differences on specific growth rate, cell concentration, amino acid consumption/production nor carbohydrates consumption rates were found at DOT between 10 and 30%. The specific growth rate and specific oxygen uptake rate followed typical Monod kinetics with respect to DOT. The calculated μ_{max} and q_{O_2} max were 0.033 h^{-1} and 3.82 × 10^{-10} mole $cell^{-1}h^{-1}$, respectively, and the corresponding saturation constants were 1.91 and 1.57%, respectively. In all aerated cultures, lactate was consumed only after glucose and fructose had been exhausted. The yield of lactate increased with decreasing DOT. It is proposed, that an 'apparent' DOT in non-instrumented cultures can be inferred from the lactate yield of bioreactors as a function of DOT. Such a concept, can be a useful and important tool for determining the average dissolved oxygen tension in non-instrumented cultures. It was shown that the dynamic behavior of OUR can be correlated with monosaccharide (fructose and glucose) depletion and viable cell concentration. Accordingly, OUR can have two important applications in insect cell culture: for on-line estimation of viable cells, and as a possible feed-back control variable in automatic strategies of nutrient addition.

Abbreviations: DOT – Dissolved oxygen tension; OUR – Oxygen uptake rate; q_{O_2} – specific oxygen uptake rate; μ – specific growth rate; X_v – viable cell concentration; C_L, C^*, and C_{H_2O} – oxygen concentrations in liquid phase, in equilibrium with gas phase, and medium molar concentration, respectively; H – Henry's constant; k_La – volumetric oxygen transfer coefficient; P_T – total pressure; p_{O_2} – oxygen partial pressure, y_{O_2} – oxygen molar fraction; i – discrete element.

Introduction

The insect cell culture-baculovirus expression vector system has been widely used for the production of proteins that require an eucaryotic expression system. In order to obtain the maximum yield of protein, a high cell concentration with optimum viability is required. For such a purpose, optimal culture environment and adequate culture monitoring are essential conditions.

In animal cell culture, dissolved oxygen tension (DOT) has been found to be one of the most critical parameters. Likewise, oxygen uptake rate (OUR) has been shown to be an effective monitoring variable for establishing control strategies. To date, several reviews have appeared describing the effect of DOT and the use of OUR in animal cell culture (Fleischaker and Sinskey, 1981; Butler and Jenkins, 1989; Thomas, 1990; Konstantinov *et al.*, Taticek *et al.*, 1994; Zhou and Mulchandani, 1995). However, only scarce and

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scattered information is available on the effect of DOT and the utility of OUR in insect cell culture.

Dissolved oxygen is significant because it tightly regulates cell physiology. Maintaining it at an adequate level, without damaging the fragile cells, also requires special considerations. This issue is of particular relevance in large-scale cultures. The effect of DOT on insect cell culture has been commonly evaluated by comparing spinner flasks with bioreactors operated at a single constant DOT (Scott *et al.*, 1992; Reuveny *et al.*, 1993; Tsao *et al.*, 1996). Such studies have reported differences in cell yields, lactate production and protein productivities between both systems. The observed differences have been attributed to the limiting DOT in spinner flask cultures, though the fact that no DOT measurements have been performed in most of these studies. Few and opposing reports exist on the comparison of cell physiology in bioreactors at various constant DOT. For instance, no significant differences in the growth and maximum viable cell concentration were found in *Bombyx mori* cells in the range of 10 to 60% DOT (Zhang *et al.*, 1994) and in Sf9 cells in the range of 5 to 100% DOT (Hensler and Agathos, 1994). In contrast, other authors have observed a decreasing growth rate and maximum cell concentration below or above an optimum DOT value, which occurred between 40 and 70 % (Klöppinger *et al.*, 1990; Jain *et al.*, 1991; Deutschmann and Jäger, 1994). Likewise, whereas Hensler and Agathos (1994) found no differences in the specific rates of oxygen (q_{O_2}) and glucose consumption in Sf9 cells between 5 to 100% DOT, Deutschmann and Jäger (1994) observed a maximum in such variables for Sf21 cells at a DOT of 70%. Moreover, Jain *et al.* (1991) reported a steady increase in q_{O_2} as DOT increased from 10 to 110%. To our knowledge, no information is available on the effect of DOT in the production or consumption of the amino acids and carbohydrates present in insect cell culture media. Furthermore, low DOT ranges (i.e., below 10%) have been scarcely studied, which are precisely where the strongest dependency of stoichiometric and kinetic variables should be expected.

Oxygen uptake rate has been found to be useful in insect cell culture for two reasons. Firstly, it has been well documented that q_{O_2} increases by as much as two-fold upon viral infection (Schopf *et al.*, 1990; Jain *et al.*, Kamen *et al.*, 1991, Scott *et al.*, 1992; Reuveny *et al.*, 1993, Hensler and Agathos, Wong *et al.*, 1994; Kioukia *et al.*, 1995; Kamen *et al.*, 1996). Secondly, Wong *et al.* (1994) and Kamen *et al.* (1996) have shown that there exists a direct relation between OUR and viable cell concentration; a relation that has been well documented for mammalian cells (Fleischaker and Sinskey, 1981; Hu and Oberg, 1990; Konstantinov *et al.*, 1994). Accordingly, q_{O_2} and OUR can be used as good process parameters for monitoring the infection phase and cell concentration, as long as continuous on-line determinations are performed. Nevertheless, most reports on OUR rely on either off-line determinations or discontinuous data obtained from the dynamic method (see Table 1). Such determinations are inadequate for automatic control algorithms and can perturb the cells due to the frequent manipulation of the culture and the oscillating DO conditions. Notable exceptions are the reports of Kamen *et al.* (1991 and 1996) for insect cells, and Ramírez and Mutharasan (1990) and Eyer *et al.* (1995) for hybridomas, where continuous and on-line OUR measurements were performed. In mammalian cell culture, on-line measurement of OUR has found an additional important application: as a feedback control variable in nutrient feeding schemes (Hu and Oberg, Ramírez and Mutharasan, 1990; Higareda *et al.*, 1993; Eyer *et al.*, Zhou *et al.*, 1995). For instance, as reported by Ramírez and Mutharasan (1990) and Higareda *et al.* (1993), OUR can be used to infer the time of glucose and glutamine depletion, and thus can be employed in nutrient feeding strategies for improving productivity. The use of such strategies prevents over- or under-feeding of key nutrients. This reduces the accumulation of toxic metabolic by-products and avoids substrate limitation. As a result, improvements by as much as one order of magnitude in monoclonal antibody productivity by hybridoma culture have been obtained. Nevertheless, to our knowledge, OUR has not been explored in insect cell culture as a tool for assessing nutrient depletion, and thus, as a possible convenient control variable in nutrient feeding schemes.

In this work, the effect of constant DOT in the range of 0 to 30%, on the growth and metabolism of Sf9 insect cell culture was studied. Consumption profiles of glucose, fructose, sucrose, and oxygen, and the consumption or production of all the amino acids, lactate, and ammonia, are reported. It is shown that continuous on-line OUR measurements can be used to assess the time of nutrient depletion. The utility of such measurements in nutrient feeding strategies, and the use of the yield of lactate on glucose for estimating an apparent DOT in non-instrumented cultures, is discussed.

Table 1. Specific oxygen uptake rate data reported in literature for insect and mammalian cell lines

Reference	Culture media	Specific oxygen uptake rate $\times 10^{-10}$ mmole cell^{-1}h^{-1}	Method
Maiorella *et al.*, 1988	IPL-41	1.5	Respirometer (off-line)
Schopf *et al.*, 1990	TNM-FH	1.4–2.2	Respirometer (off-line)
Kamen *et al.*, 1991	IPL-41	2	Gas phase balance
King *et al.*, 1992	Sf900	1.9–2	Dynamic
Scott *et al.*, 1992	Sf900	2.9	Respirometer (off-line)
Reuveny *et al.*, 1993	ICSF-WB	1.9–4	Respirometer (off-line)
Deutschmann and Jager, 1994[a]	IPL-41	0.3–1.4	Dynamic
Hensler and Agathos, 1994	Excell-401	3.3	Dynamic
Wong *et al.*, 1994	Sf900	2.2	Dynamic
Kioukia *et al.*, 1995	TC100	1.3	Respirometer (off-line)
Kamen *et al.*, 1996	Sf900	1.5[c]	Liquid phase balance
This work	TNM-FH	0.7–3.6	Liquid phase balance
Eyer, 1995[b]	Various	0.4–5	Various (mostly off-line and dynamic)

All values reported for Sf9 cells, except [a], for Sf21 cells and [b], for various mammalian cell lines. Values for insect cells correspond to non infected cultures.
[c] Calculated from data.

Materials and Methods

Cell line and culture medium

The Sf9 cell line, at an unknown passage number, was used in this study. All experiments were initiated with cells in passages between 10 to 30 of the original unknown passage number. Cell stocks were kept in 100-ml (60-ml working volume) spinner flasks at 27 oC and agitated at 100 rpm with a suspended magnetic bar impeller placed at 0.3-cm from the bottom. TNM-FH medium (Sigma Chemical Co., T-1032), supplemented with 10% (v/v) fetal bovine serum (Sigma Chemical Co., F-2449) of the same lot number and 0.05% (w/v) of Pluronic F-68 (BASF), was used in all cultures. The pH was adjusted with NaOH to give a final value of 6.2 after medium filtration. Bioreactor cultures were supplemented with 1% antibiotic antimycotic solution (Sigma Chemical Co., A-9909, Penicillin 10,000 U l^{-1}, streptomycin 100 mg l^{-1}, and amphotericin B 250 μg l^{-1}). Exponentially growing cultures were centrifuged to eliminate spent medium, and were used as inoculum for all experiments. All cultures were initiated at a viable cell concentration of 0.2 x 10^6 cell l^{-1}.

Bioreactor cultures

A 1.7-L Virtis baffled-bioreactor (10.5-cm diameter), with a working volume of 700-mL, maintained at 100 rpm and 27 oC, was used in this study. The bioreactor was equipped with two impellers: a 4.3-cm diameter suspended magnetic bar, placed 2-cm from the bottom; and a 6-cm diameter 4-blade impeller, placed at the liquid surface. The latter impeller was used to improve the oxygen transfer coefficient, since oxygen was provided only by surface aeration. Such a configuration resulted in a volumetric oxygen transfer coefficient (k_La) of 2.4 h^{-1}, which varied less than 5% throughout the culture. The pH and DOT were measured with sterilizable glass and polarographic probes (Ingold), respectively. A gas mixture of oxygen or air and nitrogen, at a constant total flow rate of 600 or 800 ml min^{-1} was delivered through the liquid surface to all cultures. In the culture at 0% DOT, only nitrogen was introduced into the bioreactor. Two mass flow controllers (Brooks Instruments, 5850E) were used to measure and control the individual flow rates of oxygen or air and nitrogen. The signals of the mass flow controllers, pH and DOT probes were amplified and acquired by a Macintosh LC computer via a MacADIOS 411 interface (GW Instruments) and a data acquisition and control program written in QuickBasic® (Microsoft) (Aguilar-Aguila *et al.*, 1993). DOT was controlled using a pro-

portional algorithm, which varied the individual gas flows via the mass flow controllers. OUR was determined on-line from the DOT and gas composition data according to a liquid phase balance described in the Results and Discussion section. Static 75-cm^3 (40-ml initial volume) T-flasks and 100-ml (60-ml initial volume) spinner flasks at 100 rpm were conducted as control cultures.

Analytical methods

Cell concentration was determined using a Coulter Multisizer II (Coulter Electronics) and cell viability by trypan blue exclusion. Glucose and lactic acid were determined enzymatically with a YSI analyzer model 2700 (Yellow Springs Instruments). Sucrose was determined using the YSI analyzer, after its hydrolysis to glucose and fructose with β-fructosidase (Boehringer Mannheim, 104914). Fructose was determined with an enzymatic kit (Boehringer Mannheim, 716260). In some experiments, fructose and sucrose concentration were only determined in every other sample. Amino acids and ammonia were determined in cell-free samples, after deproteinization with 5-sulfosalicilic acid, in a Beckman Amino Acid Analyzer model 6300. A 10-cm single ion-exchange column for lithium buffer methods (Beckman, 338051) with ninhydrin detection, was used.

Results and discussion

On-line determination of OUR

An oxygen balance in the liquid phase yields the following equation:

$$\frac{dC_L}{dt} = k_La\,[C^* - C_L] - [q_{O_2}X_v] \qquad (1)$$

where C_L and C^* are the oxygen concentrations in the liquid phase and in equilibrium with the gas phase, respectively, and Xv is the viable cell concentration. Taking discrete steps of equation 1 yields:

$$OUR = q_{O_2}X_v = \frac{k_La}{2}\,[(C_i^* + C_{i+1}^*) - (C_{L_i} + C_{L_{i+1}})] - \frac{[C_{L_{i+1}} - C_{L_i}]}{[t_{i+1} - t_i]} \qquad (2)$$

where i is the discrete element. If a high oxygen flow rate – compared to the oxygen uptake rate – and a well-mixed gas phase in the headspace are maintained, then the oxygen partial pressure (p_{O_2}) in the inlet stream will be approximately equal to the outlet p_{O_2}. Due to the low OUR reached in insect cell cultures (less than 0.5 mmol l^{-1} h^{-1} at 10^6 cells ml^{-1}; see Table 1), it can be shown that the steady-state difference between inlet and outlet p_{O_2} will be within less than 0.5%, if a ratio of oxygen flow rate to reactor headspace volume is maintained above 0.1 min^{-1}. Furthermore, for the gas mixing process in the headspace, a total gas flow rate to reactor headspace volume above 0.6 min^{-1} results in a first order time constant (characteristic time scale that defines the dynamic behavior of a first order process) of less than 2 min (Stephanopoulos, G., 1984). Compared to the time scale of insect cell culture (which is in the order of days), such a time constant is negligible. Therefore, by assuming oxygen equilibrium between the gas and liquid phases and maintaining the conditions mentioned above, C^* can be calculated from the inlet oxygen partial pressure according to:

$$C^* = \frac{[C_{H_2O}P_T y_{O_2}]}{H} = \frac{[C_{H_2O}p_{O_2}]}{H} \qquad (3)$$

where C_{H_2O}, P_T, y_{O_2}, and H are the medium molar concentration (assuming water), total pressure, oxygen molar fraction in the gas phase, and Henry's constant for oxygen in medium at 27 °C, respectively. Henry's constant was taken as 45,587 atm, from interpolation of data reported by Liley *et al.* (1984). The p_{O_2} was obtained from the ratio of oxygen to total flow rates.

Utility of on-line OUR measurements in Sf9 cell culture

Typical results of an Sf9 batch culture controlled at 10% DOT are shown in Figure 1. It can be seen that, during the first 4 days, OUR closely followed the exponential increase in viable cell concentration (Fig. 1A). Accordingly, OUR can be used as a convenient variable for on-line estimation of viable cell concentration during exponential growth phase. The direct correlation between cell density and OUR can be clearly seen in Fig. 2. Such a correlation has been well documented for mammalian cell cultures (Fleischaker and Sinskey, 1981; Hu and Oberg, 1990; Konstantinov *et al.*, 1994), but has only been recently reported for insect cell culture (Wong *et al.*, 1994; Kamen *et al.*, 1996). As described in the following section, q_{O_2} becomes a function of DOT for values below 10%. Thus, only the data for the 10% and 30% DOT cultures were plotted in Fig. 2.

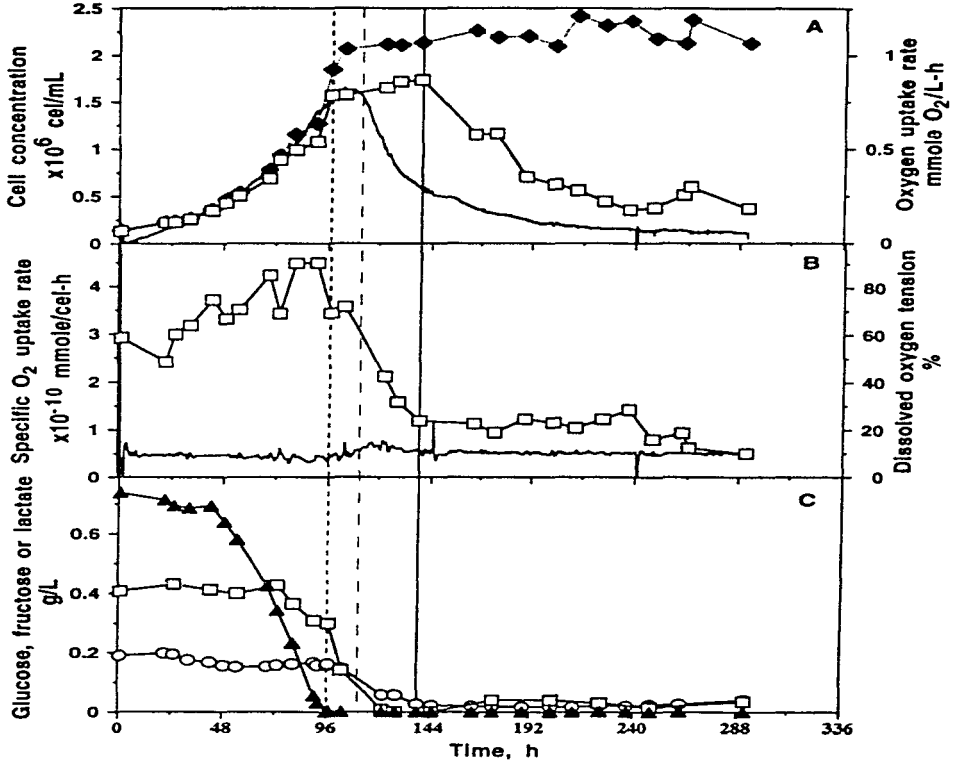

Figure 1. Typical behavior of a batch culture at 10% DOT. A, viable cell concentration (□), total cell concentration (◆) and OUR (—). B; specific oxygen uptake rate (□) and DOT (—). C, glucose (▲), fructose (□) and lactate (○). The vertical dotted-line denotes the time of glucose depletion and the end in rise of OUR, the dashed-line, the time of the onset of respiration cessation; and the solid line, the time of lactate depletion and the end of the stationary growth phase.

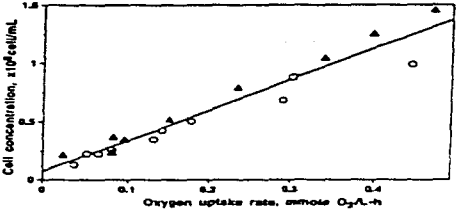

Figure 2. Viable cell concentration, during exponential growth of Sf9 cultures, is linearly related to OUR. The equation obtained from bioreactor runs at 30 (▲) and 10 (○) % DOT was $X_v = 2.651\ OUR + 0.073$, with a correlation coefficient (r^2) of 0.937

As shown in Table 1, most of the existing reports have determined OUR by either off-line methods or by dynamic measurements. However, as discussed by Yoon and Konstantinov (1994) and Eyer *et al.* (1995), such methods can present important disadvantages. For instance, off-line methods are labor intensive, can hardly be incorporated into automatic real-time monitoring and control algorithms, and can cause culture failure due to increased manipulation. Dynamic measurements expose the cultures to undesirable DOT fluctuations, and are difficult to automate due to the multiple-successive tasks required. In addition, dynamic measurements and off-line methods do not provide a continuous signal, and only a few data points per day can be collected. Therefore, rapid phenomena, as described below, can occur undetected when relying on such measurements. Methods based on gas phase measurements can overcome the above limitations. However, expensive equipment, such as mass spectrometers are necessary. Nevertheless, due to the low OUR of animal cell cultures, it is usually difficult to accurately detect the small difference between the inlet and outlet oxygen concentration (Zhou and Mulchandani, 1995). In contrast, as seen in Fig. 1A, the method presented here provides continuous on-line OUR data, relies on relatively inexpensive equipment, and can be readily automated. It should be noted that this method has been used before in mammalian cell cultures (Ramírez and Mutharasan, 1990; Higareda *et al.*, 1993; Eyer *et al.*, 1995), and only recently in insect cell cultures (Kamen *et al.*, 1996).

As seen in Fig. 1B, the control algorithm used in this work proved to be an accurate method for maintaining a constant DOT. Similar good control was obtained for the other DOT tested, even at 2% DOT, where accurate control is usually difficult to attain (Court, 1988). In contrast, methods based only on enriching oxygen in the gas phase are unable to control the DOT during the phases of low OUR, i.e., during the lag and death phases (Hensler and Agathos, 1994).

During exponential growth, q_{O_2} remained relatively constant at a high value of $3.6 \pm 0.6 \times 10^{-10}$ mmole cell^{-1} h^{-1}, and glucose was preferentially used over fructose and lactate (Figs. 1B and 1C). Upon glucose limitation (< 0.2 g l^{-1}) fructose consumption began, whereas lactate uptake occurred only after glucose depletion. The end of the exponential growth phase and the end in rise of OUR occurred upon glucose exhaustion (indicated by the dotted-vertical line in Fig. 1), even though fructose was still present at 0.3 g l^{-1}. In addition, cell viability started to decrease upon glucose depletion. Interestingly, the onset of a rapid decrease in OUR occurred 12 hours after glucose depletion and when fructose concentration had decreased below 0.15 g l^{-1} (dashed vertical line). In some cases, glucose depletion was enough to mark OUR decrease and thus, from here on, we will refer to glucose and fructose as monosaccharides. A similar behavior in OUR was observed for all batch cultures above 2% (data not shown). Nonetheless, cells remained in stationary phase for an additional day, until lactate was completely consumed (solid vertical line). The depletion of this carbon source coincided with the sharp decrease in viability. The cessation in respiration caused by the depletion of a key nutrient, namely glucose or glutamine, has been previously demonstrated in hybridoma cell culture (Ramírez and Mutharasan, 1990; Higareda *et al.*, 1993; Eyer *et al.*, 1995). However, to our knowledge, this is the first report that correlates nutrient depletion with respiration cessation in insect cell culture.

A similar sequence of carbon source uptake, as described in Fig. 1, was observed for all batch cultures above 5% DOT. Furthermore, only cystine and glutamine were completely exhausted during all batch cultures, either at the end of the exponential growth phase or at the stationary phase (data not shown). However, the time of their depletion did not coincide with the sharp decrease in OUR. Accordingly, the characteristic behavior of growth cessation and sudden decrease in OUR and q_{O_2} in batch cultures, succeeding glucose and fructose depletion, clearly indicates that such monosaccharides are the main carbon sources and are metabolized to produce energy via oxidative phospho-

Table 2. Specific oxygen uptake rate before and after monosaccharide depletion at different DOT and in a fed-batch culture

Dot %	Specific oxygen uptake rate x 10^{-10} mmole cell^{-1} h^{-1}	
	Before monosaccharide depletion	After monosaccharide depletion
30	2.55	1
10	3.56	1.2
5	3.22	1.6
2	2.06	1.5

rylation. Furthermore, neither growth nor OUR could be sustained by the concentration of lactate, amino acids or sucrose present following monosaccharide depletion. In Table 2 it is shown that an important decrease in q_{O_2} (and thus OUR) occurs upon monosaccharide depletion for a broad range of DOT tested. Thus, OUR can be used as an on-line parameter for estimating depletion of such a nutrient. Ramírez and Mutharasan (1990) and Higareda *et al.* (1993), have shown similar results for hybridoma cell culture, where Gln or glucose depletion could be detected on-line by changes in OUR. Overall, the results shown in Fig. 1 demonstrate that OUR measurements are useful not only for estimating viable cell concentration during exponential growth phase, but are also important for assessing the growth phase stages and determining the time when key nutrients become limiting or are exhausted. This is particularly relevant, since OUR could then be employed as a simple and convenient variable in feed-back control algorithms for improving productivity through automatic nutrient addition strategies.

Effect of DOT on growth and metabolism

The growth kinetics for batch cultures maintained at constant DOT in the range of 0 to 30% are shown in Figure 3. It should be noted that no reports were found of insect cell cultures maintained at a constant DOT below 5%. No significant differences on cell growth and maximum viable cell concentration were observed for cultures maintained between 5 and 30% DOT. For the culture at 2% DOT, compared to those at 5% DOT and higher, specific growth rate was about 50% lower, whereas maximum viable cell concentration was about 30% higher. Duplicate cultures showed the same differences in maximum X_v between

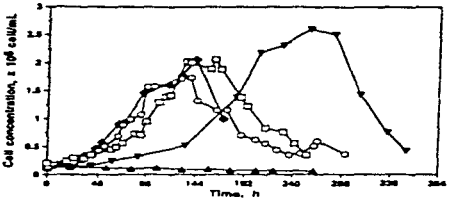

Figure 3. Cell growth kinetics of Sf9 at 30 (□), 10 (○), 5 (◆), 2 (▼) and 0 (▲) % DOT. No difference was observed for DOT Above 2%

the 2% DOT and other aerated cultures. No explanation could be found for such differences from the metabolites measured in this study. Therefore, different consumption/production patterns of organic acids, could be responsible for such behavior, but this was not tested in this work. Bédard *et al.* (1993) have reported significant changes in the concentration of organic acids, in addition to lactate, during Sf9 cultures in TNM-FH medium. In the culture maintained under anaerobic conditions no growth was observed, and the viable cell concentration slowly declined as time progressed. This is consistent with Rhiel and Murhammer (1995), who reported growth inhibition of Sf21 cells when exposed to short (30 min) anaerobic periods, and with Wang *et al.* (1993), who observed growth cessation as oxygen was depleted in a non-DOT controlled bioreactor.

A summary of monosaccharides consumption and lactate production/consumption as a function of DOT is shown in Fig. 4. In all cultures, glucose was found to be the main carbon source, which is in accordance to previous reports (Bédard *et al.*, 1993). Except for the cultures below 2% DOT, fructose consumption began after glucose concentration decreased below 0.2 g l^{-1}. For the 2% DOT culture, fructose and glucose consumption occurred simultaneously. Lactate was produced in cultures below 10% DOT. In the culture at 5% DOT, a net lactate production of 0.06 g l^{-1} was observed during the exponential growth phase. This value is higher than the observed experimental error, which was typically less than +/- 0.003 g l^{-1}. Lactate was consumed after monosaccharide depletion in all the aerated cultures. Such a behavior has been observed by others (Kamen *et al.*, 1991; Bédard *et al.*, 1993), and contrasts with the general behavior of many mammalian cells. That is, in mammalian cell culture, lactate

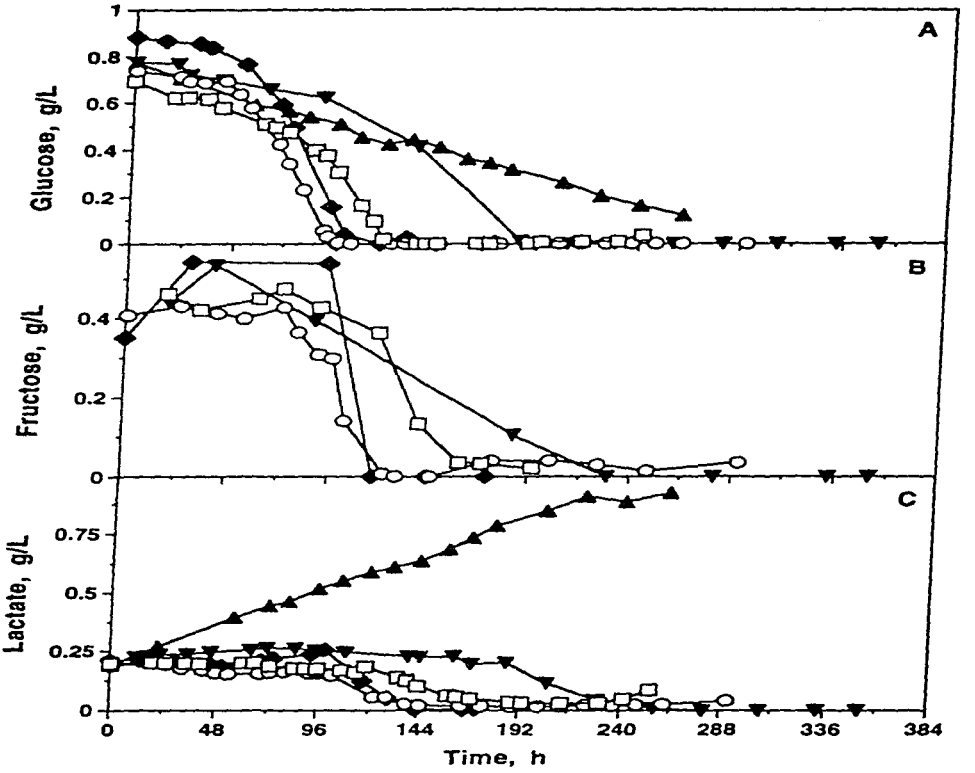

Figure 4. Effect of DOT on: A, glucose; B, fructose and C, lactate production or consumption kinetics. Legend as in Figure 3. Fructose was not measured for the anaerobic culture. No significant variations in sucrose were observed in any of the DOT tested (data not shown).

is generally recognized as a toxic by-product which is not usually consumed (Ozturk *et al.*, 1992). In the aerated experiments, pH remained relatively constant at around 6.2 during the growth phase, and increased by no more than 0.7 units during the death phase. Such an increase is most probably due to the observed lac-

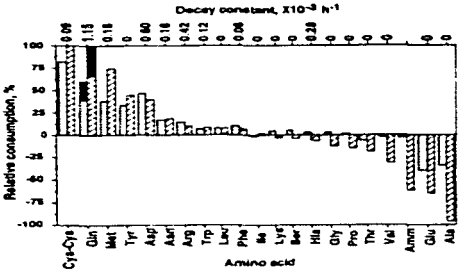

Figure 5. Relative consumption of amino acids (with respect to initial composition) during Sf9 batch culture at 30% DOT, at glucose depletion (dotted bar) or at the end of the culture (striped bar). For glutamine, the total bar height represents the observed disappearance, whereas the solid bar is the calculated percentage corresponding to the spontaneous decomposition. For the rest of the amino acids, the spontaneous decomposition was less than 3%. The first order decay constants, in h^{-1}, are shown at the top of the figure.

tate consumption. In anaerobic conditions, lactate was produced during the entire culture period, even when no cell growth was observed. Nevertheless, viable cell concentration remained at about 0.12×10^6 cell ml^{-1} for more than six days, which explains the fermentation of the carbon source to lactate. For such cultures, the calculated maintenance coefficient (assuming that only 2 ATP moles are produced per mole of glucose consumed) was 2.53×10^{-10} mmole ATP $cell^{-1} h^{-1}$, which agrees well with those reported for hyridoma culture (Miller *et al.*, 1989). We have not found a toxic effect of lactate, even at concentrations two-fold above the maximum observed in anaerobic conditions (data not shown). Accordingly, the growth inhibition in such a culture cannot be attributed to lactate. No variations in sucrose concentration were observed for all DOT tested (data not shown). Sucrose concentration has also been reported to remain constant for cultures under different DOT conditions (Reuveny *et al.*, 1992; Bédard *et al.*, 1993; Drews *et al.*, 1995).

The relative consumption or production of amino acids and ammonia, illustrated in Fig. 5 for a 30% DOT culture, showed the same general features for all DOT tested. In order to determine the extent of spontaneous decomposition of all amino acids, cell-free culture medium was incubated at 27 °C and first order decay constants were calculated. Only Gln presented a significant spontaneous decomposition (almost 30%), indicated by the solid bar in Fig. 5, which corresponded to a decay rate constant of $1.15 \times 10^{-3} h^{-1}$. The spontaneous decomposition of other amino acids was less than 3%, and their decay constants are shown in the top of Fig. 5. In Fig. 5, the moment of glucose depletion (dotted-bars) and the end of the culture (striped bar) were taken as arbitrary times for facilitating comparison. As seen, the amino acids can be classified in three groups according to their consumption or production profiles at the end of the culture. Group A includes amino acids that were consumed in more than 25% with respect to their initial concentrations (cystine, Gln, Met, Tyr, Asp). Group B includes those that were produced in more than 25% with respect to their initial concentrations (Val, Ala, Glu), and group C includes all others, which did not change more than 25%. Gln has been reported to be essential for insect cells (Mitsuhashi, 1982), however it did not limit growth in the aerated cultures as its depletion occurred only after the stationary phase had been reached. Cystine is also essential for Sf-9 cell growth (Tremblay *et al.* 1992), and its depletion has been correlated with the induction of apoptosis in mammalian cells (Mercille and Massie, 1994). Nevertheless, due to analytical limitations, most literature reports do not present data on cystine. As discussed previously, the depletion of cystine in batch cultures did not coincide with the cessation of growth and respiration, which was caused by monosaccharide depletion. Interestingly, cystine depletion for cultures above 5% DOT occurred during late exponential phase, whereas for the 2% DOT culture it occurred during the end of the stationary phase. This indicates that the observed cessation of growth and respiration cannot be attributed to a delayed effect of cystine depletion, as has been reported for other animal cells (Mercille and Massie, 1994).

If comparison is made at the time of glucose depletion, only Ala, and Glu were produced at significant extent, while all the other amino acids were either consumed or remained relatively constant. Compared to previous reports, two noteworthy differences were found. Whereas a consistent production of Ala and Glu was observed in this work, others (Bédard *et al.*, 1993; Ohman *et al.*, 1995) have reported, for Sf9 cells, a production of Ala only until glucose depletion, and a rapid consumption of Glu. Nonetheless, it has been reported that Glu can be produced in insect cell culture from Asp, Gln, Ala or Pro (Mitsuhashi, 1982). For all DOT tested, the highest Ala concentration obtained

was 13 mM. Alanine has been generally considered to be a toxic by-product in animal cell cultures. However, the concentration reached in this work was most probably not inhibitory, since Bédard *et al.* (1994) and Hensler and Agathos (1994) have proven that alanine is not toxic to insect cells, even at 40 mM. An average Glu concentration of 4 mM was observed in all batch cultures, which corresponded to an increase of about 60% with respect to the initial concentration. In comparison, Mitsuhashi (1982), has reported a 20% increase on Glu concentration in *Papilio xuthus* ovary cells cultures.

From inoculation and throughout the exponential growth phase, ammonia concentration remained relatively constant at about 2 mM in all DOT tested. During the stationary and death phases, ammonia concentration increased, but never exceeded a final concentration of 4 mM. As Gln decomposition and metabolism of amino acids yields ammonia, the results observed indicate that a net ammonia consumption was occurring. Such a behavior has been reported previously by Bédard *et al.* (1993), Drews *et al.* (1995), and Ohman *et al.*, (1995), and is thought to be due to ammonia incorporation to yield Ala. The final concentration of ammonia attained in all batch cultures was not toxic to Sf9 cells in TNM-FH medium, as we have found no effect in growth rate even at ammonia concentrations of 15 mM (data not shown).

A summary of the effect of DOT on kinetic and stoichiometric parameters is shown in Fig. 6. The data points for 2, 10 and 30% DOT correspond to the mean of duplicate cultures. Error bars have been included, but in some cases were smaller than the plot symbols. For q_{O_2}, the values correspond to the exponential growth phase, and the error bars correspond to the standard deviation. It can be seen that maximum cell concentration, specific growth rate (μ), and q_{O_2}, remained relatively constant for DOT in the range of 5 to 30%. X_v values obtained in this work are comparable with those reported in literature (Murhammer and Goochee, 1988; Oogonah *et al.*, 1991; Drews *et al.*, 1995). A constant maximum X_v, μ, and q_{O_2} have been reported for *B. mori* and Sf9 cells in a range of 10 to 60% and 5 to 100% DOT, respectively (Hensler and Agathos; Zhang *et al.*, 1994). In contrast, an optimum DOT between 40 and 70%, with decreasing X_v, μ, and q_{O_2} at higher or lower DOT has also been reported (Kloppinger *et al.*, 1990; Jain *et al.*, 1991; Deutschmann and Jäger, 1994). Other contrasting results are those of Jain *et al.* (1991), who have observed a steady increase in q_{O_2} as DOT increased from 10 to 110% in infected cultures. As seen in Fig. 6A, the highest maximum cell concentration was obtained in the culture at 2% DOT. No cell growth was observed in the anaerobic culture, and the maximum cell concentration corresponded to the inoculum.

As shown in Figs. 6B and 6C, μ, and q_{O_2} followed a Monod-type behavior with respect to DOT, where the continuous lines correspond to the model simulation. The calculated μ_{max}, and q_{O_2} max were 0.033 h^{-1} and 3.82 X 10^{-10} mmole $cell^{-1}$ h^{-1}, respectively, and the corresponding calculated saturation constants were 1.91% and 1.57%, respectively. To our knowledge, no reports exist for insect cells at constant DOT values below 5%. Accordingly, the Monod constants reported here represent the only quantitative parameters available. The q_{O_2} values observed in this work compare very well with those reported for insect and mammalian cell lines (see Table 1).

The yield of lactate produced per glucose consumed was determined during the exponential growth phase, and is shown in Fig. 6D. There was no production of lactate in cultures at 30 and 10% DOT, and the initial lactate concentration due to serum, was consumed throughout the cultivation period. Accordingly, the negative yield values refer to lactate consumption. Surprisingly, Deutschmann and Jagger (1994) have reported a considerable lactate production at 40% DOT in Sf21 cells. As DOT decreased from 5 to 0%, the lactate yield increased from 0.11 to 2.4 mole/mole. It is widely recognized that lactate is produced in insect cell culture during oxygen limitation (Wang *et al.*, 1993; Bédard *et al.*, 1994; Rhiel and Murhammer, 1995). However, most studies reporting lactate production have been performed in non-instrumented flasks. In such cultures it can only be assumed that oxygen limitation exists, since no dissolved oxygen measurements can be performed. We propose that an 'apparent' DOT in non-instrumented cultures can be inferred from interpolation of the lactate yield profile of bioreactors as a function of DOT. Since DOT will constantly decrease in non-controlled cultures, 'apparent' DOT refers to the value at which equivalent stoichiometric and kinetic parameters, with respect to controlled cultures, would be obtained. In Fig. 6, the maximum X_v, μ, and lactate yield obtained in T-flasks and spinner flasks have also been plotted as the horizontal-dotted lines in the corresponding figure panels. If vertical lines are drawn, by intersecting the lactate yield of T-flasks and spinner flasks with the interpolated values obtained in bioreactors, one can determine an apparent DOT. As seen, for T-flasks, the apparent DOT is around 1.5%,

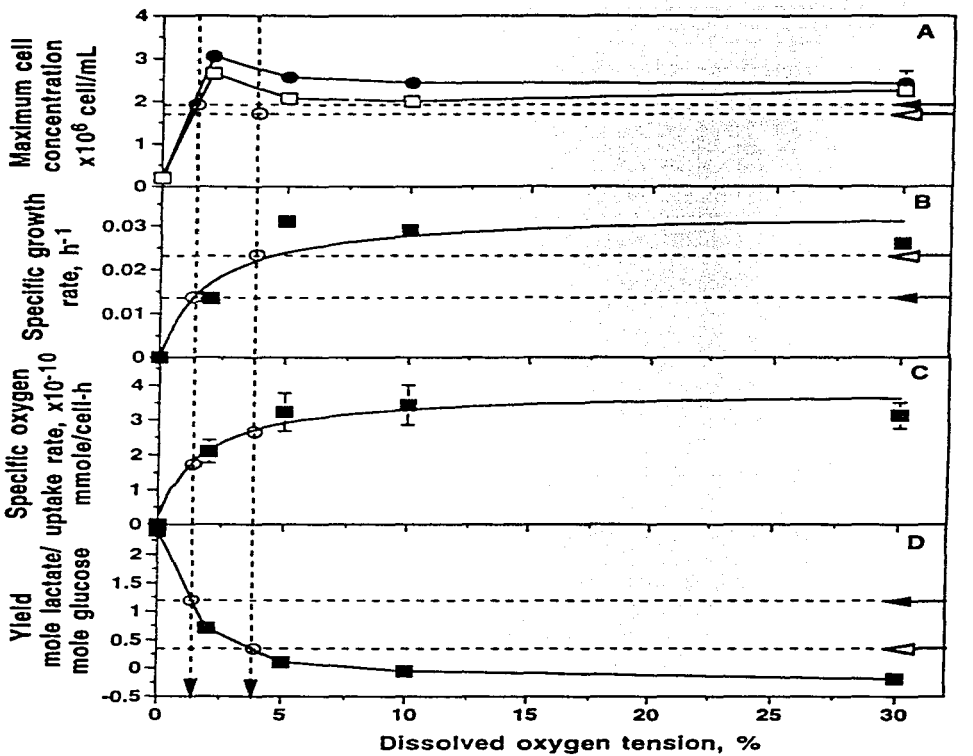

Figure 6. Effect of DOT on maximum cell concentration, specific growth rate, specific oxygen uptake rate and yield of lactate on glucose. A: maximum total (●) and maximum viable cell concentration (□). B, the continuous line represents the Monod model simulation, where K_{O_2} is 1.91 % and μ max is 0.033 h^{-1}. C, Mean specific oxygen uptake rate during the exponential phase. The continuous line represents the Monod model simulation, with a K'_{O_2} of 1.57 % and a q_{O_2} max of 3.82 X 10^{-10} mmole $cell^{-1}h^{-1}$. The error bars in C correspond to the standard deviation in q_{O_2} observed during exponential growth phase. Cultures at 2, 10 and 30% DOT were performed in duplicate. Some error bars are smaller than the symbols shown. The dotted-horizontal lines represent the experimental values obtained in spinner flasks (open arrows) and in T-flasks (closed arrows). The vertical-dotted lines correspond to the apparent DOT of T-flasks and spinner flasks. The open circles indicate the intersection of the calculated parameter with the apparent DOT.

while in spinner flasks, the apparent DOT is around 4%. Furthermore, the experimental maximum X_v and μ of T-flasks (1.8 X 10^6 cell ml^{-1} and 0.014 h^{-1}, respectively) and the experimental μ of spinner flasks (0.024 h^{-1}), agree very well with the interpolated values from the apparent DOT (intersections of vertical- and horizontal-dotted lines, open circles). In turn, the maximum experimental X_v in spinner flasks (1.8 X 10^6 cell ml^{-1}) was within 20% error with respect to the expected value obtained from the apparent DOT. The use of the 'apparent' DOT concept, proposed here, can be a useful and important tool for determining the average DOT in non-instrumented cultures.

Conclusions

The effect of constant DOT, in the range from 0 to 30%, on growth and metabolism of Sf9 cells in batch culture was evaluated. No effect was observed at DOT in the range of 10 to 30%. Thus, a strict DOT control in such a range is not required. For cultures at DOT below 10%, the yield of lactate on glucose increased with decreasing DOT. This relation can be used to estimate an 'apparent' DOT in non-instrumented cultures (T-flasks and spinner flasks), which are usually limited by oxygen. This concept can be useful as an indirect experimental tool, since it can be employed to accurately predict the X_v and μ of such cultures. In all cultures, glucose was the main carbon and energy source, as observed from the growth and respiration cessation upon its depletion, and its consumption profiles. After glucose became limiting, fructose and lactate were consecutively utilized. The concentration of sucrose did not change throughout the various cultures, and cystine and glutamine were the only amino acids completely consumed. Finally, it was shown that an oxygen balance in the liquid phase can be used, together with relatively inexpensive experimental equipment, for on-line OUR determination. Such a method, offers substantial advantages over other traditional techniques for determining OUR. It was shown that the dynamic behavior of OUR can be correlated with nutrient depletion and viable cell concentration. Accordingly, OUR can have two important applications in insect cell culture: for on-line estimation of viable cells, and as a possible feed-back control variable in automatic strategies of nutrient addition.

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FALTAN PAGINAS.

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V.4.2 Monitoreo y control en línea de cultivos infectados

Como ha sido discutido en las **Secciones V.3** y **V.4.1**, un abastecimiento adecuado de nutrimentos antes y después de la infección es indispensable para lograr buenos rendimientos y productividades del sistema. Para lograr ésto se requuiere tener un entendimiento completo del metabolismo celular antes y después de la infección. Varios grupos han trabajado en lograr éste entendimiento, lo que ha resultado en diferentes estrategias para lograr un abastecimiento adecuado de nutrimentos después de la infección, que incluyen la perfusión, recambio de medio y alimentación de nutrimentos (Bédard et al., 1993; 1994; Wang et al., 1993; Caron et al., 1994, Öhman et al., 1995, 1996; Radford et al., 1997; Chan et al., 1998). Sin embargo, las estrategias a seguir deben estar basadas en el entendimiento del metabolismo celular después de la infección, y apoyadas por estrategias adecuadas de monitoreo y control. Con el fin de ampliar el conocimiento del papel de los nutrimentos y de evaluar la utilidad de la velocidad de consumo de oxígeno (VCO) para monitorear cultivos después de la infección, se realizaron varios cultivos en biorreactores instrumentados descritos en la **Tabla V.1**.

Tabla V.1 Descripción de los cultivos en biorreactor y resumen de la producción de proteína recombinante.

Figura	Biorreactor	Nutrimentos añadidos al infectar[a]	Otras adiciones[a]	VP8 max U/mL	Rendimiento x10^{-6} U/cel
V.1	0.7L			16.9	9.7
V.2	3.8L	Glc		285.93	95.59
	3.8L	Glc+Gln		293.27	129.61
V.3	0.7L	Glc	1. Glc* 2. Gln*	278.54	128.3

* Basada en la VCO

a. En todos los casos se alimentaron glucosa (Glc) y Gln para lograr una concentración final de 0.6 g/L o 1.6 mM, respectivamente.

La **Figura V.1** muestra un cultivo en lote realizado en un reactor instrumentado de 1L. Como muestra la línea punteada, el reactor fue infectado a las 73h de cultivo. En el primer panel de la figura se muestra la concentración celular, el perfil de la VCO y del oxígeno disuelto (OD). Como se puede inferir por el perfil de la VCO (ver **Sección V.4.1**), y como se observa en el panel intermedio, los monosacáridos habían sido totalmente consumidos antes de la infección. La VCO permaneció constante después de la infección, hasta que se inició la lisis celular. La VCO trazó de cerca el perfil de la concentración celular viable después de infectar. La concentración máxima de VP8 obtenida fue de 16.9 U/mL, y se detectó a las 96 h de cultivo (ver **Tabla V.1**).

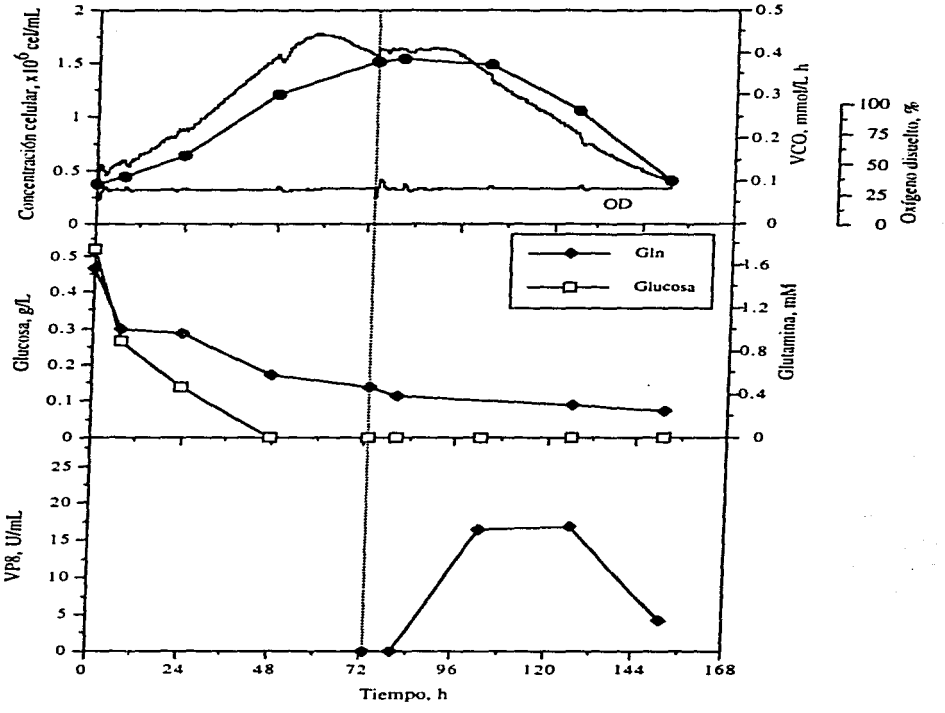

Figura V.1 Cultivo lote en reactor instrumentado. OD se refiere a oxígeno disuelto. La línea punteada marca el momento de la infección con bac8 a una MDI de 10 ufp/cel.

Un segundo cultivo, mostrado en la **Figura V.2**, fue alimentado con glucosa al momento de infectar. En contraste con el cultivo lote, la adición de virus y glucosa provocó un aumento de la VCO del 80%, comparado con la VCO al momento de infectar. La adición de únicamente glucosa a cultivos no infectados no provoca un aumento en la VCO (datos no mostrados), por lo que el comportamiento observado debe ser consecuencia de la infección. Varios grupos han

observado incrementos en la VCO al momento de infectar de hasta el 100%, lo que se atribuye a un aumento en el metabolismo celular después de la infección (Kamen et al., 1991, 1996; Hensler y Agathos, 1994; Wong et al., 1994; Cruz et al., 1998). Sin embargo, en éste trabajo no se observó un aumento en las velocidades específicas de consumo de glucosa o de glutamina a la par con el aumento de la VCO (3.63 $x10^{-10}$ mmol carbono/cel h y 0.34 $x10^{-10}$ mmol/cel h, respectivamente, después de infectar. Comparar con **Sección V.3**).

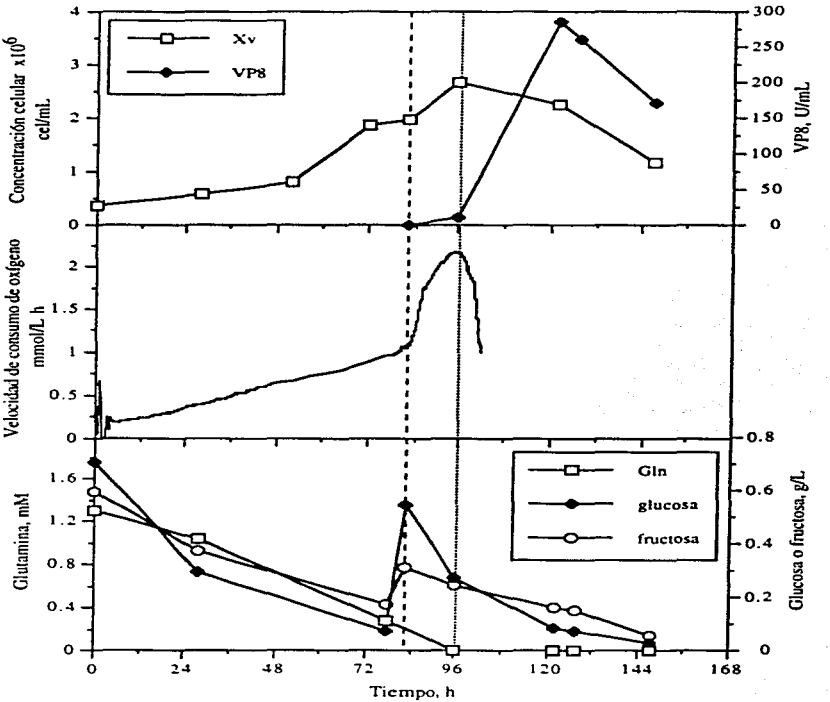

Figura V.2 Cultivo alimentado con glucosa al momento de infectar. La línea interrumpida señala el momento de infección. La línea punteada señala el momento del agotamiento de la Gln.

Hasta el momento, ninguno de los grupos de investigación mencionados ha reportado cuál es el nutrimento que sostiene el aumento en la VCO, aunque Wong et al. (1994) suponen que se trata de un aminoácido. Fue muy interesante observar que la infección del cultivo lote no provocó un aumento en la VCO. Esto demuestra que la presencia de glucosa es indispensable para que exista el aumento en la VCO, aunque su consumo específico no se incrementa. Anteriormente, otros grupos han reportado la ausencia del aumento de la VCO después de la infección en algunos cultivos con bajas productividades, lo que se ha atribuido a una infección ineficiente (Kamen et al., 1991, 1996). Sin embargo, este trabajo es el primero en demostrar que la glucosa es esencial para que aumente la VCO, y que, más que una infección ineficiente, la falta de éste incremento tiene un origen nutrimental. En concordancia con los otros reportes de la literatura sobre cultivos donde no aumentó la VCO al infectar, el cultivo lote produjo 16 veces menos VP8 que el cultivo V.15 (ver **Tabla V.1**).

A las 96 h de cultivo se observó una drástica caída en la VCO, que además coincidió con el inicio de la fase de muerte del cultivo (**Figura V.2**). El análisis de las muestras mostró que la caída en la VCO coincidió con el agotamiento de Gln en el cultivo. Como se discute en la **Sección V.4.1**, un comportamiento similar ha sido observado en cultivos de hibridomas (Ramírez y Mutharasan, 1990; Eyer et al., 1995; Higareda et al., 1997). En el caso de cultivos infectados de células de insecto, hasta éste trabajo la caída en la VCO no había sido relacionada con algún evento en el cultivo. El que el agotamiento de la Gln provoque una drástica caída en la VCO parece indicar que se trata de la principal fuente para la fosforilación oxidativa (ver **Sección V.3**). Después de las 100h de cultivo fue imposible controlar el OD, por lo que no se pudo estimar la VCO.

La VCO, como se ha discutido en la **Sección V.4.1**, ha sido utilizada como una herramienta poderosa para monitorear el agotamiento de nutrimentos en cultivos de células de mamíferos y diseñar estrategias de alimentación. Con el fin de determinar si es posible implementar éste tipo de estrategias en cultivos infectados de células de insecto, se realizó un cultivo igual al mostrado en la **Figura V.2**, pero adicionando Glc y Gln conforme se detectó una caída en la VCO (ver también **Sección V.4.1**). Las cinéticas del cultivo se muestran en la **Figura V.3.** Los monosacáridos, como señala el perfil de la VCO, se agotaron a las 75 h de cultivo. Por lo tanto se alimentó glucosa junto con el virus. En ésta ocasión la infección provocó un aumento del 60% en la VCO. Como se puede observar en el perfil del OD, algunas perturbaciones en el control introdujeron ruido en la estimación de la VCO. Para discriminar entre las perturbaciones y una disminución real de la VCO, es necesario considerar también el perfil del OD: cuando se trata de una perturbación, el perfil del oxígeno disuelto aumentará como consecuencia de un cambio en la composición de los gases a la entrada. Cuando se trata

de un disminución en la VCO, el OD aumentará a pesar de que la composición de los gases a la entrada sea constante. De esta forma, es posible discriminar entre eventos operacionales y eventos metabólicos en los cultivos.

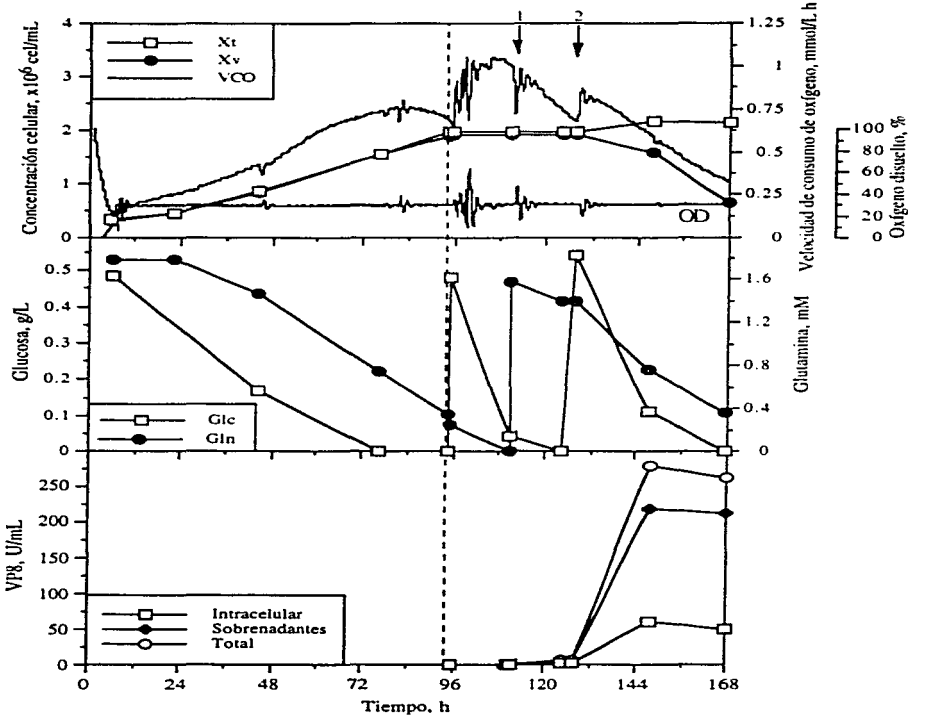

Figura V.3 Cultivo alimentado con base en la VCO. La línea interrumpida señala el momento de infección y alimentación de Glc. 1 y 2 se refieren a los momentos de adición de Gln y Glc, respectivamente.

A las 110 h se detectó una drástica caída en la VCO, de pendiente muy similar a la observada cuando se agotó la Gln el cultivo anterior. Por lo tanto se alimentó Gln. En el panel intermedio se observa que la caída en la VCO coincidió con el agotamiento de la Gln. La alimentación provocó una recuperación en la VCO al nivel que tenía antes del agotamiento de la Gln. Posteriormente se observó una caída gradual de la VCO, que, con base en los resultados mostrados en la **Sección V.4.1**, corresponde al comportamiento observado cuando se agotan los monosacáridos. La glucosa fue entonces añadida al cultivo, lo que provocó una recuperación de la VCO. En la **Tabla V.2** se muestra una comparación entre las velocidades de disminución de la VCO conforme el sustrato agotado. Cuando se agotan los monosacáridos la VCO disminuye con una velocidad 22 veces menor que cuando se agota la Gln. Esto pone claramente en evidencia el diferente papel de cada uno de estos nutrimentos en el metabolismo de la célula infectada, uno como principal sustrato para la respiración, y otro como principal fuente de esqueletos carbonados, como se ha discutido también en la **Sección V.3**. Además, permite discriminar claramente cuál fue el sustrato agotado, y tomar una acción correctiva en caso necesario.

Tabla V.2 Velocidad de disminución de la VCO provocada por el agotamiento de Glc o Gln en los cultivos. Se muestra media y desviación estándar de las caídas de la VCO observadas en todos los cultivos.

Nutrimento agotado	$\frac{dVCO}{dt}$, $\frac{nmol}{x10^6 cel \cdot h^2}$
Glucosa	-7.5 ± 1.38
Glutamina	-168 ± 32

Como se muestra en la **Tabla V.1**, la alimentación con base en la VCO no incrementó la concentración de proteína recombinante, aunque el rendimiento por célula aumentó un 34%. Como control, se realizó otro cultivo en el que se alimentaron tanto Glc como Gln al momento de infectar. Los resultados obtenidos se muestran para comparación en la **Tabla V.1**. Dicho cultivo tuvo un rendimiento muy similar al cultivo alimentado con base en la VCO. Esto confirma la importancia de un abastecimiento adecuado de Gln y Glc al momento de infectar.

Aunque la alimentación con base en la VCO no presentó ventajas sobre la adición de nutrimentos al momento de la infección, ésta herramienta sigue siendo útil para monitorear los cultivos, y tomar alguna acción correctiva en caso de alguna eventualidad operacional que provocara un agotamiento prematuro de los nutrimentos (adición de menor cantidad de nutrimentos, o subestimación de las necesidades del cultivo).

V.5 Producción de las proteínas recombinantes de rotavirus por células de insecto.

El primer paso para lograr un entendimiento de la producción de las proteínas multiméricas es obtener los parámetros cinéticos de producción de cada una de las diferentes proteínas que conforman las PPV. Esta información permitirá no solo el entender la dinámica de producción de los diferentes monómeros de proteínas, sino que además será posible identificar los pasos limitantes para la producción de proteínas recombinantes y, como consecuencia, diseñar estrategias que agilicen estos pasos. Para definir la estrategia experimental se partió de un análisis del proceso de infección basado en información de la literatura. La infección de células de insecto por baculovirus sigue una distribución de Poisson (de Gooijer et al., 1989; Licari y Bailey, 1992; Tsao et al., 1996). Por lo tanto, es posible predecir la cantidad de células que serán inicialmente infectadas en un cultivo, de acuerdo con la MDI, si se supone que la infección por cada virus es un evento independiente, y que la infección primaria es simultánea. La probabilidad de que una célula sea infectada por w virus estará dada por:

$$p(w) = \left[\left(\frac{MDI^{w}}{w!} \right) e^{-MDI} \right] \qquad V.1$$

Utilizando la ec. V.1, se puede predecir la probabilidad de que una célula sea o no infectada por una o más partículas virales, como se muestra en la **Figura V.4**. Es claro que, para que prácticamente el 100% de la población sea infectada por lo menos por un virus, se debe utilizar una MDI $\geq$ 5 ufp/cel.

Con base en esta información, se decidió utilizar una MDI de 5 ufp/cel para determinar las velocidades de producción de las proteínas recombinantes a partir de una población prácticamente 100% infectada. Los experimentos se realizaron en frascos agitados utilizando medio SF900II y una densidad celular al momento de la infección de $1x10^6$ cel/mL. Se determinó que las infecciones realizadas en estas condiciones no sufren limitación por nutrimentos o por oxígeno (datos no mostrados). En la **Figura V.5** se muestra una cinética típica de producción de proteína recombinante, ejemplificada con el caso de VP7. La cinética sigue el comportamiento esperado para cultivos infectados en condiciones similares y expresando proteínas de rotavirus, tal como ha sido reportado previamente (Labbé et al., 1991; Sabara et al., 1991; Bédard et al., 1994; Wong et al., 1996). La producción de proteína recombinante fue detectable después de las 24 horas postinfección (hpi), y continuó hasta el final del cultivo. Cabe aclarar que, aunque en general las cinéticas observadas para la producción de

cada una de las cuatro proteínas recombinantes fue similar, el sitio y la velocidad de expresión de las proteínas fueron muy diversos, como se muestra en la **Tabla V.3**. VP2 se encontró en todos los casos en la fracción insoluble de las células, en concordancia con reportes anteriores (Labbé et al., 1991). VP6 se encontró asociada a las células excepto cuando la viabilidad celular empezó a descender. Aparentemente, se expresa en la fracción citoplásmica. Esto concuerda con el reporte de Sabara et al. (1991) de que no hay liberación de VP6 hacia el exterior de las células, aún cuando la viabilidad haya disminuido drásticamente. VP7 fue encontrada en los sobrenadantes aún desde las 48 hpi (**Figura V.5**). Ya que VP7 esta glicosilada, existe la posibilidad de que sea excretada por las células. Este comportamiento concuerda con el observado por Sabara et al. (1991).

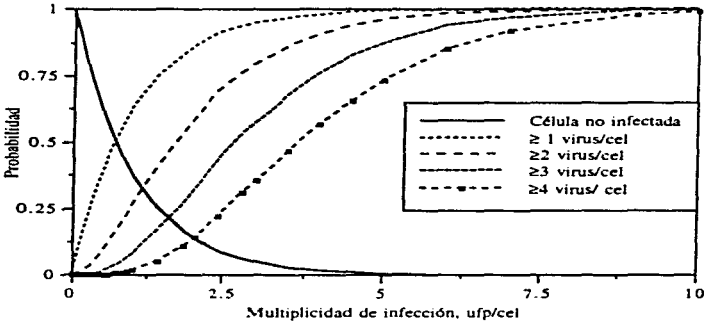

Figura V.4 Probabilidad de infección según la distribución de Poisson.

Tabla V.3 Parámetros cinéticos de producción de proteínas a MDI = 5 ufp/cel y TDI = $1x10^6$ cel/mL. En el caso de VP2, VP6 y VP7, media y desviación estándar de 3 experimentos. Las velocidades de expresión se calcularon a partir de la pendiente entre la concentración de proteína recombinante y el tiempo entre las 18 y las 55 hpi. Para calcular las velocidades específicas se dividió la velocidad de expresión entre la concentración celular promedio durante ese tiempo.

Proteína	**VP2**	**VP6**	**VP7**	**VP4**
Sitio de expresión	membrana	citoplasma	secretada	membrana
q Proteína U/mL h	0.58±0.1	7±1.5	13.2± 3	0.96
q Proteína U/10^6 cel h	0.48±0.11	6.5 ± 1	19.46± 5	0.82
Proteína max U/mL	34.95±3	358.8 ± 14	698 ± 19.5	39.4

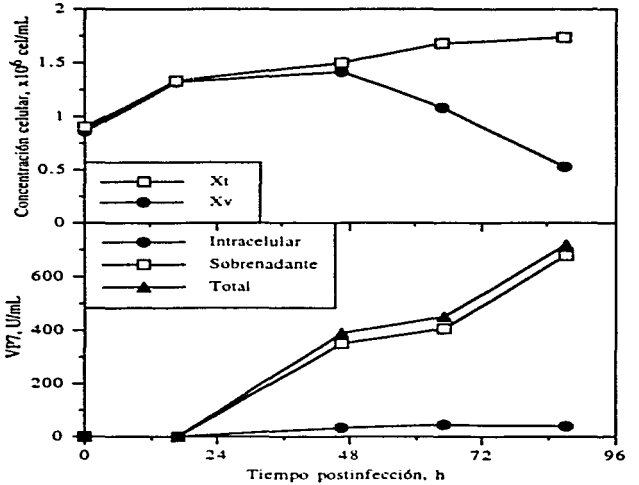

Figura V.5 Cinética típica de un cultivo infectado a $1x10^6$ cel/mL y con una MDI de 5 ufp/cel.

Resultó muy interesante observar una correlación logarítmica entre la velocidad de expresión y el tamaño de las proteínas recombinantes (**Figura V.6**). Es importante tomar en cuenta que las velocidades observadas de producción de las proteínas recombinante son producto tanto de las velocidades de producción como de las de degradación. Por lo tanto, las velocidades reales de producción de proteínas probablemente serán mayores a las reportadas en este trabajo. Aparentemente el tamaño de cada una de las proteínas determina su velocidad de síntesis, independientemente de la existencia de modificaciones postraduccionales. La relación entre el tamaño y la concentración máxima de cada proteína puede ser consecuencia de estas diferencias entre las velocidades de síntesis, ya que la fase de producción de proteína recombinante presumiblemente dura lo mismo en cultivos infectados en las mismas condiciones (O'Reilly et al., 1994).

Con el fin de explicar este comportamiento, se realizó un análisis cinético del proceso de producción de proteínas a partir de información de la literatura. La velocidad de producción de las proteínas depende de la velocidad de síntesis de los mensajeros, la velocidad de traducción de

los ribosomas, el número de ribosomas realizando la traducción y la velocidad de degradación de las proteínas y de los mensajeros (Farewell y Neidhardt, 1998).

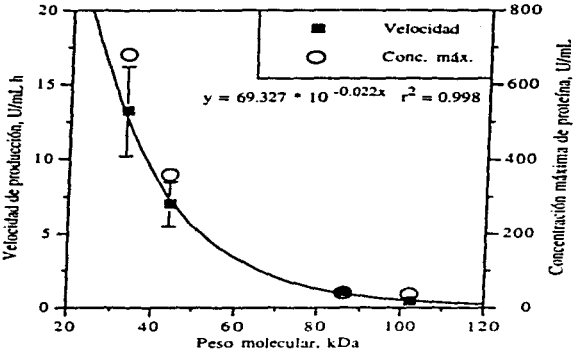

Figura V.6 Relación entre el peso molecular y la velocidad de producción de proteínas recombinantes en células de insecto. Se muestra la correlación entre la velocidad de producción y el peso molecular. Los símbolos y las barras representan la media y desviación estándar de tres experimentos, respectivamente, excepto para VP4. En algunos casos las barras son más pequeñas que el símbolo (ver **Tabla V.3**).

Cuando existen condiciones de exceso de nutrimentos y las condiciones ambientales son las adecuadas (pH, OD, etc.), la velocidad de transcripción de la RNA polimerasa y de traducción de los ribosomas presumiblemente son constantes y las máximas (Chohji et al., 1976; Mowbray y Nierlich, 1975). Ya que los genes que codifican para cada una de las proteínas recombinantes se encuentran bajo el mismo promotor, y fueron producidas bajo las mismas condiciones (MDI, TDI, etc.), se puede asumir que las velocidades de traducción de los ribosomas y de transcripción de las polimerasas fueron iguales para los 4 casos (Whiteley et al., 1997). Igualmente, podemos suponer que las frecuencias de iniciación de la traducción y la transcripción y la degradación de mensajeros fueron iguales e independientes del tamaño de cada proteína (Bibila y Flickinger, 1991; Whiteley et al., 1997). Aunque aún no se ha determinado la naturaleza de la RNA polimerasa responsable de la transcripción de mensajeros bajo el promotor de la polihedrina (O'Reilly et al., 1994), supondremos que dicha polimerasa transcribe a una velocidad similar a la reportada para otras polimerasas de células eucariotes superiores cultivadas *in vitro*, 83 nucleótidos/pol s (Bibila y Flickinger, 1991). La velocidad de transcripción estará dada por:

$$\frac{dARN_m}{dt} = \frac{v_{pol}N_{pol}}{L_{ARN_m}} \qquad V.2$$

donde N_{pol} es en número de polimerasas trabajando, v_{pol}, la velocidad de transcripción de cada polimerasa y L_{ARNm}, la longitud del ARN_m.

De igual forma, es posible calcular la velocidad de traducción de las proteínas recombinantes con base en la velocidad de traducción de un ribosoma (v_{rib} = 20 nucleótidos/ribosoma s), y el número de ribosomas unidos a cada transcrito, N_{rib}, 1 ribosoma/135 nucleótidos (Bibila y Flickinger, 1991):

$$\frac{dprot}{dt} = \frac{v_{rib}N_{rib}N_{ARN_m}}{L_{ARN_m}} \qquad V.3$$

donde N_{ARNm} es el número de mensajeros disponibles para la traducción. Utilizando las ecs. V.2 y V.3 se generó la **Tabla V.4**.

Tabla V.4 Parámetros cinéticos calculados a partir de información de la literatura.

Proteína	Aminoácidos[a]	Nucleótidos/ mol ARN_m	Velocidad de transcripción mol ARN_m/pol s	Ribosomas/ transcrito	Velocidad de traducción, mol/ARN_m s
VP2	880	2640	0.0314	19	0.1439
VP4	776	2328	0.0357	17	0.1460
VP6	397	1191	0.0697	8	0.1343
VP7	326	978	0.0849	7	0.1431

a. Mattion et al., 1994.

Es claro que, aparentemente debido a que mensajeros más largos son simultáneamente traducidos por una mayor cantidad de ribosomas, la velocidad de traducción no guarda la relación observada entre el peso molecular y la velocidad de producción de proteínas en la **Figura V.6**, mientras que la velocidad de transcripción si la guarda, como se muestra en la **Figura V.7**. Los cálculos apoyan la hipótesis de que la velocidad de transcripción sea el paso limitante para la producción de las diferentes proteínas recombinantes. Esto se refuerza con el hecho de que la velocidad de transcripción resultó ser, a lo más, el 60% de la velocidad de traducción. Experimentos posteriores, que serán discutidos más adelante, también apoyan esta hipótesis.

V.6 Uso de la MDI como herramienta para manipular la velocidad de producción y la concentración de proteínas recombinantes.

Se infectaron cultivos con diferentes MDI, para explorar la utilidad de esta variable para manipular la velocidad de producción de proteínas recombinantes. Como se muestra en la **Figura V.8**, un incremento en la MDI de 1 a 5 ufp/cel incrementó un 80 % la velocidad de producción de VP2. Cuando se utiliza una MDI de 1 ufp/cel, únicamente el 63% de la población sería infectada al momento de agregar el virus (ver **Figura V.4**). El resto de la población se infectaría con la progenie viral producida por las células inicialmente infectadas (infección secundaria). Esto explica la reducción en la velocidad de producción de proteínas. De la misma forma, la concentración máxima de VP2 aumentó un 25% al aumentar la MDI de 1 a 5 ufp/cel.

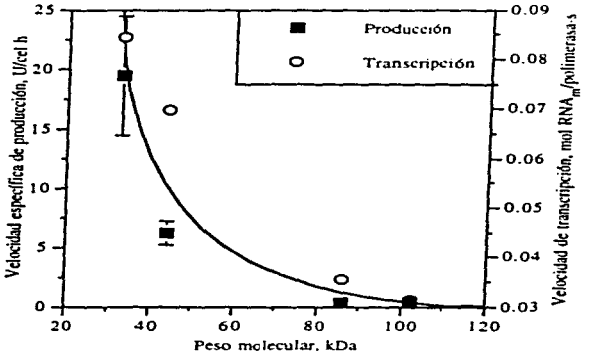

Figura V.7 Comparación entre las velocidades específicas de producción de proteínas obtenidas experimentalmente con las velocidades de transcripción calculadas. Para los datos experimentales, las barras indican desviación estándar de tres experimentos, excepto para VP4. En algunos casos las barras son más pequeñas que el símbolo (ver **Tablas V.4** y **V.5**). La línea se muestra únicamente para indicar la tendencia.

En el caso de los cultivos infectados con 5 y 10 ufp/cel, el 100% de la población sería infectada al momento de agregar el virus, y la diferencia consiste en el número de viriones que infectan cada célula. En la **Figura V.4** se observa que a una MDI de 10 ufp/cel, prácticamente el 100% de la población sería infectada por más de 3 virus/cel, mientras que a una MDI de 5 ufp/cel, el 73% de la población estaría infectada por más de 3 virus/cel. Esto se vería reflejado en un incremento en el número de templados de ADN disponibles para la transcripción, y en un

consecuente aumento en la velocidad de producción de proteína recombinante. El aumento en el número de viriones que infecta a cada célula se vió reflejado en un aumento del 75 ± 5% en la velocidad específica de producción de VP2. Puede esperarse que este efecto tenga un comportamiento de saturación, donde, a partir de que los templados de ADN estén en exceso, un aumento en la MDI ya no tendrá un efecto en la velocidad de producción de proteínas. Esa es la tendencia que se observa en la **Figura V.8**, y coincide con el comportamiento observado en la literatura en cultivos infectados con hasta 100 ufp/cel (Licari y Bailey, 1991; Wong et al., 1996). No hubo una diferencia significativa entre las concentraciones máximas de VP2 alcanzadas a MDI de 5 y 10 ufp/cel. Es claro que la MDI puede ser utilizada como herramienta para manipular la velocidad, y por lo tanto la concentración máxima, de las proteínas recombinantes. Esta herramienta será útil para lograr la estequiometría correcta entre las varias proteínas cuando se pretenda producir multímeros.

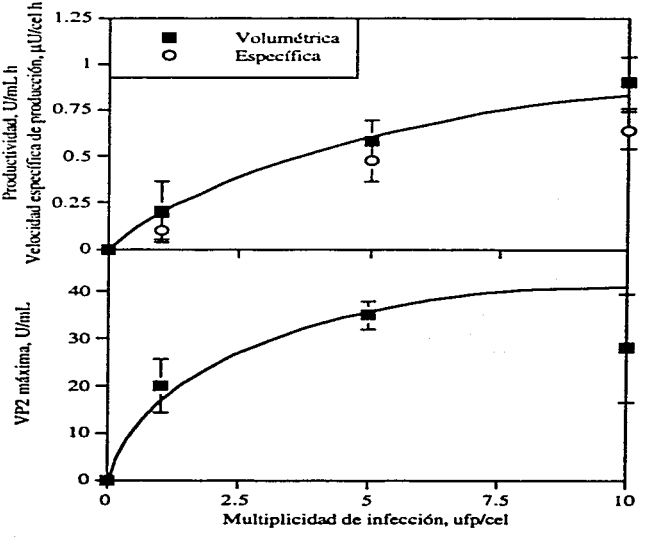

Figura V.8 Efecto de la MDI sobre la velocidad de producción y concentración máxima de VP2. Las barras muestran la desviación estándar de tres experimentos. La línea únicamente muestra la tendencia.

V.7 Producción simultánea de VP2 y VP6.

La infección de cultivos con un solo tipo de virus puede generar una gran cantidad de poblaciones: células no infectadas, infectadas por 1, 2, etc. viriones (ver **Figura V.4**). El sistema se vuelve aún más complejo cuando se realizan coinfecciones con 2 o más virus diferentes. Además de las poblaciones ya enumeradas, se tendrá que considerar las células infectadas solo por 1 tipo de virus, por el segundo tipo de virus, por ambos tipos, y diferentes números de cada virus. Lógicamente, la producción de las diferentes proteínas recombinantes será el reflejo de estas poblaciones. Para conocer el proceso de producción de dos proteínas simultáneamente, se realizaron coinfecciones con bac2 y bac6. Cuando se realizan coinfecciones con dos baculovirus diferentes, la ecuación V.1 toma la siguiente forma (Tsao et al., 1996):

$$p(w,y) = \left[\left(\frac{a^w}{w!}\right)e^{-a}\right]\left[\left(\frac{b^y}{y!}\right)e^{-b}\right] \qquad V.4$$

donde a y b son las MDI de bac2 y bac6, respectivamente, y "w" y "y" son el número de virus de bac2 y bac6, respectivamente, que infectarían cada célula. Si utilizamos una MDI de 5 para cada baculovirus recombinante (MDI total = 10ufp/cel), el 99.3% de la población será inicialmente infectada por lo menos por 1 baculovirus de cada tipo. Se escogió esta estrategia de infección para realizar una caracterización inicial de la coinfección y la producción simultánea de ambas proteínas recombinantes. Una cinética típica se muestra en la **Figura V.9**.

Tal como era de esperarse, el cultivo coinfectado se comportó de forma similar a un cultivo infectado con un solo tipo de virus a una MDI de 10 ufp/cel: prácticamente no aumentó la concentración celular después de la infección, la viabilidad del cultivo empezó a disminuir 24 h antes que en el cultivo mostrado en la **Figura V.5**, y la viabilidad diminuyó hasta el 20% también 24 h antes que el cultivo a una MDI de 5 ufp/cel, en congruencia con otros reportes de la literatura (Wong et al., 1994, Wu et al., 1993). Es importante notar que, mientras que el rotavirus nativo tiene una relación VP6/VP2 de 3.4, la relación entre las proteínas obtenida fue de 15.5. La relación entre ambas proteína es clave para el proceso de ensamblaje. Hay que recordar que las PPV son estructuras muy complejas que requieren del número exacto de monómeros de proteína para su ensamblaje. Es obvio que es necesario regular la expresión de ambas proteínas recombinantes para obtener una expresión que se equipare con la presente en el rotavirus nativo.

En la **Tabla V.5** se comparan las velocidades de expresión de ambas proteínas en cultivos coinfectados y en cultivos infectados por un solo tipo de baculovirus recombinante. No hubo diferencia significativa entre ambas velocidades. Este resultado apoya, una vez más, la hipótesis

de que son la transcripción y el número de templados de ADN disponibles, los limitantes para la velocidad producción de proteínas recombinantes. Según la información disponible, esta es la primera vez que se reportan y comparan los parámetros cinéticos de producción simultánea de 2 proteínas recombinantes por células de insecto. La importancia de estos resultados para la realización del presente trabajo es que es posible extrapolar los parámetros cinéticos obtenidos en infecciones individuales para el caso de coinfecciones, y entonces predecir el comportamiento de los cultivos coinfectados.

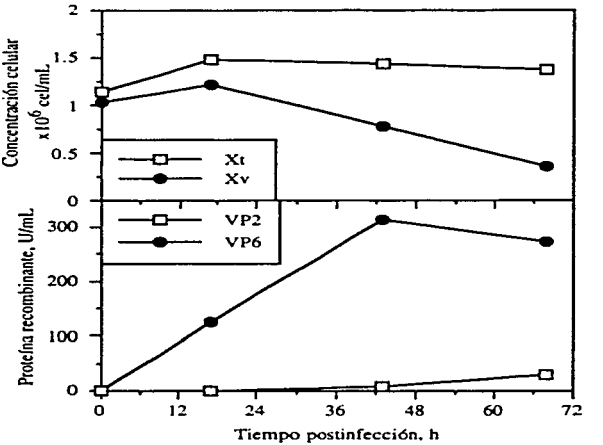

Figura V.9 Expresión simultánea de VP2 y VP6 en un cultivo infectado con una MDI de 5 ufp/cel de cada baculovirus recombinante.

Tabla V.5 Velocidad de expresión de VP2 y VP6 en cultivos coinfectados o infectados individualmente.

Tipo de expresión	Velocidad de expresión, U/mL h	
	VP2	VP6
Individual	0.58 ± 0.13	7 ± 1.5
Simultánea	0.48	7.31

V.7.1 Uso de la MDI para manipular la estequiometría entre VP2 y VP6 en cultivos coinfectados.

De los resultados obtenidos, el manipular la MDI es la estrategia lógica para obtener una determinada estequiometría entre las proteínas recombinantes. La **Figura V.10** muestra las estequiometrías entre VP6 y VP2 que resultaron de diferentes relaciones entre las MDI. Los resultados obtenidos se comparan con un cultivo infectado con un baculovirus doble, bac2/6, que codifica simultáneamente para VP2 y VP6.

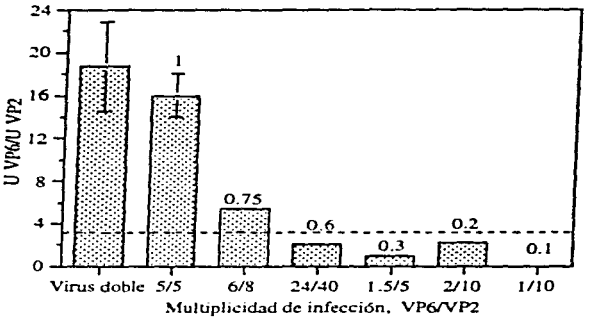

Figura V.10 Estequiometría entre VP6 y VP2 en cultivos infectados a diferentes MDI de cada virus. Los números sobre las barras indican la relación entre ambas multiplicidades. La línea punteada señala la estequiometría entre VP6 y VP2 en los rotavirus nativos, 3.4. Para el virus doble y para 5/5, las barras indican media y desviación estándar de 3 experimentos.

Es clara la utilidad de utilizar diferentes relaciones entre las MDIs para modificar la estequiometría entre las proteínas. Deben hacerse varias consideraciones importantes. Primero, que el uso de MDI altas de ambos virus asegura que cada una de las células sea infectada por lo menos por un virus de cada tipo. Esto se ha considerado importante para lograr un correcto ensamblaje de las proteínas en multímeros, como se discute más adelante (**Sección V.7**). Segundo, el utilizar MDI demasiado altas (ca. 100 ufp/cel) podría anular la utilidad de la estrategia, ya que existirían copias en exceso de ambos genes, y el efecto de las tasas entre las MDI se anularían. Finalmente, la alta imprecisión de las técnicas existentes para titular los lotes de virus dificulta utilizar esta estrategia. En este trabajo se han utilizado los mismos lotes de virus para asegurar que las diferentes relaciones entre bac2 y bac6 sean reales entre los experimentos. El utilizar virus múltiples, como el bac2/6, tiene la ventaja de que se asegura que

cada célula infectada contenga al menos una copia de cada uno de los genes de las diversas proteínas que se deseen expresar. Sin embargo, con el virus doble sería imposible manipular la relación entre protínas.

La estequiometría entre VP6 y VP2 utilizando bac2/6 y utilizando bac2 y bac6 a MDIs de 5/5 fueron muy similares, lo que coincide con lo predicho por la ec. V.1 y los resultados mostrados en la sección anterior. De igual forma, una disminución de la MDI de bac6 en relación con la de bac2, aumentó la fracción de VP2. El efecto de la MDI fue muy similar al observado en los cultivos infectados por un solo tipo de baculovirus (ver **Figura V.8**). Según la información disponible, únicamente el trabajo de Tsao et al. (1996) ha manipulado la estequiometría de producción de dos proteínas recombinantes utilizando las MDI. Utilizando esta estrategia, lograron obtener PPV de parvovirus con composición constante y altamente inmunogénicas. En contraste con las PPV de rotavirus, las PPV de parvovirus se pueden ensamblar aún con diferentes estequiometrías entre las proteínas que las conforman (Tsao et al., 1996). En el caso de las PPV de rotavirus, el poder obtener una estequiometría determinada entre las proteínas recombinantes podría resultar en un incremento de las proteínas ensambladas. Sin embargo, aunque en este trabajo no se evaluó el efecto de diferentes MDI en el ensamblaje de los multímeros, los resultados indican que para obtener una estequiometría entre VP6 y VP2 cercana a la presente en el rotavirus se deberá utilizar una proporción entre ambas MDIs de 0.6 a 0.2 ufp bac6/ufp bac2.

V.7.2 Uso del TDI para manipular la estequiometría entre VP2 y VP6 .

El otro parámetro importante en el diseño de estrategias de infección es el TDI. El TDI, como se ha explicado en los antecedentes, se refiere al momento del cultivo en el que se agrega el virus, y es determinante para laproductividad de la proteína recombinante (ver **Antecedentes**). Como se ha discutido en los **Antecedentes**, la infección de las células de insecto por el baculovirus tiene varias fases: la temprana (0-6 hpi), tardía (8-18 hpi) y muy tardía (20-72 hpi). Se ha determinado que existe unión de baculovirus a las células y transporte de nucleocápsides virales al núcleo celular aún 24 hpi (Dee y Shuler, 1997). Esto significa que las células pueden ser nuevamente infectadas hasta 24 h después de la infección inicial. Sin embargo, no se conocen las características de esta segunda infección, ni cómo sería la expresión de las proteínas recombinantes, si la segunda infección fuera producto de un baculovirus recombinante diferente al primero. Pueden plantearse dos hipótesis. Primero, que la replicación del segundo tipo de baculovirus fuera más eficiente, o por lo menos igual que la del primer tipo. Esto se esperaría en el escenario de que los segundos viriones que entren a la célula encuentren ya funcionando toda la maquinaria celular necesaria para producir las proteínas virales, como consecuencia de la

infección por el primer virus. La segunda hipótesis sería que, aún cuando la célula siga siendo susceptible a una nueva infección, esta infección no fuera tan eficiente como cuando la célula no ha sido infectada, y por lo tanto la expresión de la segunda proteína se vería disminuida de una forma similar a cuando se disminuye la MDI. Esto podría ser consecuencia de los grandes cambios en las células que provoca la infección con baculovirus en la etapa temprana: cambios en el citoesqueleto, disminución en la cantidad de receptores virales, etc. (Miller, 1996). Como primer acercamiento se trabajó con la segunda hipótesis, y se utilizó un ΔTDI (tiempo de infección con el segundo tipo de baculovirus (bac6) - tiempo de infección con el primer tipo de baculovirus (bac2)) para regular la estequiometría de producción entre las proteínas recombinantes. Según la información disponible, esta estrategia no ha sido propuesta por otros grupos de investigación. Se decidió utilizar un intervalo de ΔTDI de 0 a 6 hpi, cuando aún las células inicialmente infectadas se encuentran en la fase temprana postinfección. Se infectaron $1x10^6$ cel/mL con una MDI de 5ufp/cel de cada baculovirus, para poder discriminar entre el efecto del ΔTDI y de las MDI. Los experimentos se realizaron por triplicado, y tuvieron tendencias similares. Uno de estos experimentos se muestra en la **Figura V.11**. El cultivo control, infectado simultáneamente con bac2 y bac6, tuvo un comportamiento similar al que se muestra en la **Figura V.9**, cultivo con las mismas características. Las proteínas recombinantes fueron detectadas 24 hpi. VP6 fue detectada en el sobrenadante después de que inició la lisis celular. La concentración máxima de VP2 fue de 32 U/mL, mientras que la de VP6 fue de 350 U/mL. La relación entre VP6 y VP2 fue de 12 (ver **Figura V.12** panel inferior). Cuando se utilizó un ΔTDI de 1 h, los cultivos prácticamente no produjeron proteínas recombinantes, y no disminuyó la viabilidad celular hasta las 140 hpi, mientras que en los otros cultivos la viabilidad disminuyó a partir de las 48 hpi. Hemos observado este comportamiento en otros cultivos poco productivos, aunque hasta el momento no podemos explicarlo. Las concentraciones celulares en los cultivos infectados con ΔTDI de 3 y 6h fueron muy similares. Sin embargo, en ninguno de estos cultivos se detectó VP6 extracelular. Es importante mencionar que la técnica utilizada para cuantificar proteína recombinante en los sobrenadantes tiene una sensibilidad casi 5 veces menor que la técnica para cuantificar proteína intracelular. Por lo tanto, mientras lo mínimo detectable intracelularmente es 2.3 U/mL, en sobrenadantes es 11 U/mL, por lo que no se puede descartar la presencia de concentraciones muy pequeñas de VP6 en los sobrenadantes (≤ 11U/mL). El retraso en la infección con bac6 resultó en un retraso en la producción de la proteína recombinante. Mientras que con un ΔTDI de 0 h VP6 se detectó a partir de las 24 hpi, con un ΔTDI de 3, VP6 se detectó 12h después, y con ΔTDI de 6h, casi 36 h después. Además, la concentración máxima de VP6 disminuyó un 95% y un 96 % con ΔTDI de 3 y 6 h, respectivamente (**Figura V.12**, panel superior). Si para todos los cultivos consideramos

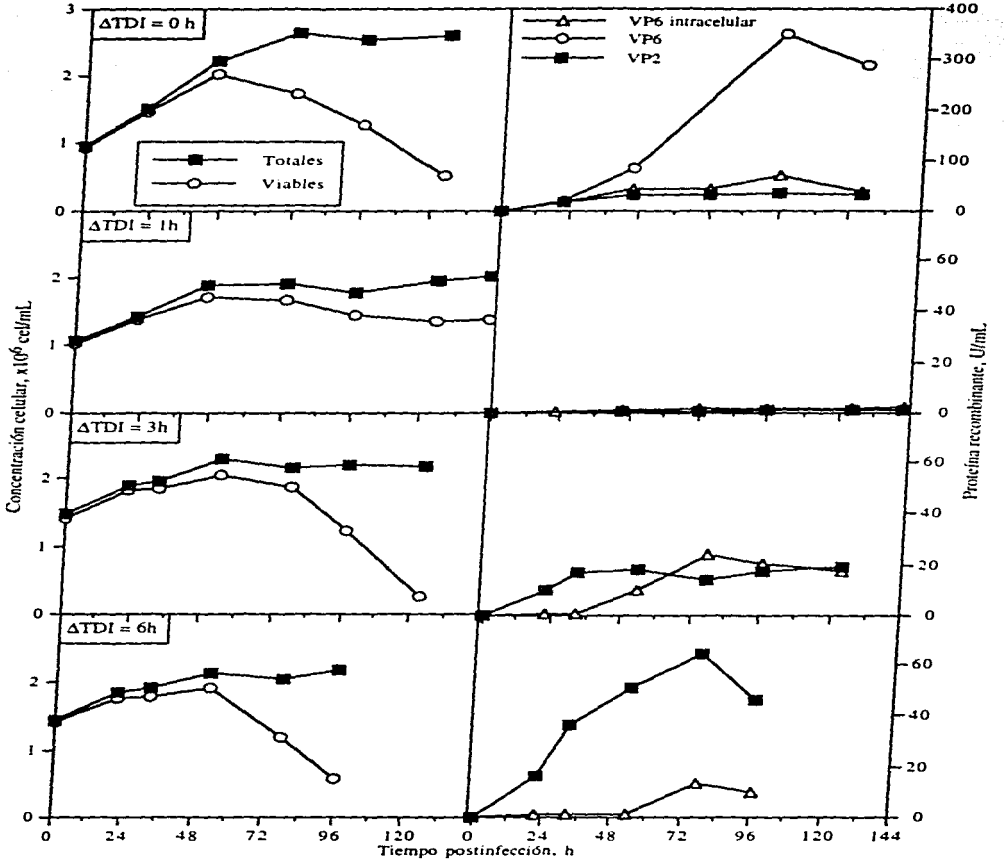

Figura V. 11 *Efecto del ΔTDI sobre las cinéticas de producción de proteínas recombinantes. Cada cultivo fue infectado con 5 ufp/cel de bac2 y bac6. VP6 se refiere a la proteína detectada intracelularmente + proteína detectada en el sobrenadante. Para los cultivos con ΔTDI >0, no se detectó VP6 en los sobrenadantes.*

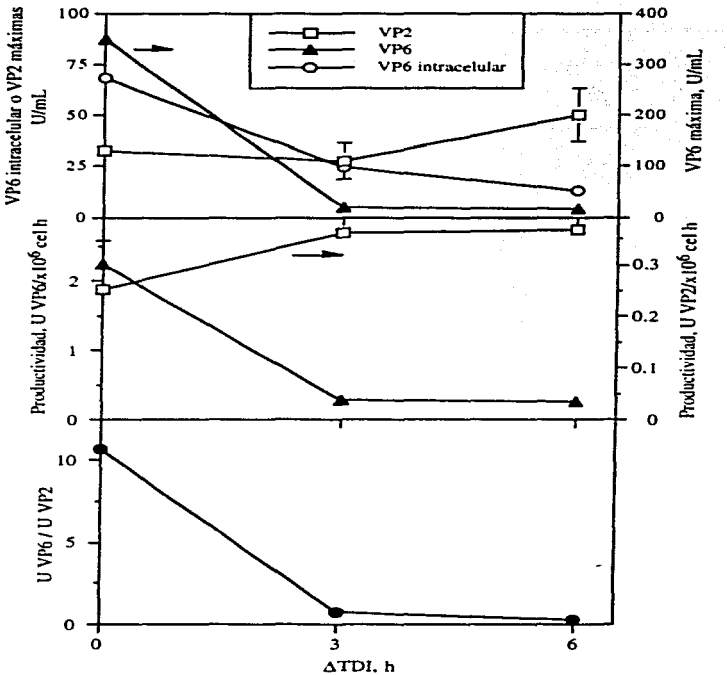

Figura V.12 Efecto del ΔTDI sobre la producción de proteínas recombinantes. Se muestra media y desviación estándar de tres experimentos. En algunos casos los símbolos son mayores que las barras de error. VP6 se refiere a la proteína detectada intracelularmente + proteína detectada en el sobrenadante. Para los cultivos con ΔTDI >0, no se detectó VP6 en los sobrenadantes.

únicamente la VP6 intracelular, entonces la disminución en la concentración máxima fue del 13 % y del 53%, respectivamente (**Figura V.12** primer panel). Mientras tanto, la concentración máxima de VP2 aumentó un 52 % en el cultivo con un ΔTDI de 6 h, en comparación con los cultivos a ΔTDI de 0 y 3h. La productividad de VP6 disminuyó un 85% con ΔTDI de 3 y 6 h, mientras que la productividad de VP2 aumentó un 45% en los mismos casos (**Figura V.12**

panel intermedio). Este comportamiento concuerda con la segunda hipótesis originalmente planteada: que el retrasar la infección con bac6 disminuiría la concentración de VP6 producida. Una consecuencia de esto fue una disminución en la estequiometría VP6/VP2 (**Figura V.12**). Esta estrategia resultó efectiva para manipular la estequiometría entre las proteínas. Para obtener una estequiometría cercana a la del rotavirus, deberá utilizarse un ΔTDI de 3h. Esta herramienta es especialmente útil, pues no requiere del uso de lotes virales con títulos muy altos, indispensables para utilizar de forma eficiente la MDI como herramienta para manipular la estequiometría entre las proteínas.

V.8 Ensamblaje de PPV.

La principal limitación para realizar un estudio cinético de la producción de PPV ha sido la dificultad para separar, identificar y cuantificar los multímeros de proteína. La técnica tradicional para realizar esto es el uso de gradientes, ya sea de CsCl o de sacarosa. Sin embargo, este procedimiento requiere de una gran cantidad de PPV, lo que únicamente se puede lograr con volúmenes de muestra muy grandes (≥100mL). Además, está técnica es costosa, muy larga y somete a la muestra a una gran cantidad de tratamientos, lo que provoca la degradación de las proteínas recombinantes y/o la ruptura de los multímeros presentes (Chen y Ramig, 1992). Los únicos 2 trabajos que, a nuestro conocimiento, han realizado estudios sobre el proceso de producción de PPV han abordado el problema de varias formas. Tsao et al. (1996) utilizaron únicamente los datos obtenidos al cosechar los cultivos. Cruz et al. (1998) utilizaron con éxito la cromatografía. El primer acercamiento tiene la desventaja de que únicamente se conoce el comportamiento del proceso en un solo punto, que pudiera no ser representativo, o pudiera variar entre diferentes estrategias experimentales. El problema se dificulta aún más cuando en los cultivos se producen simultáneamente varios tipos de PPV, y es necesario identificar y cuantificar cada uno. Esto aún no es posible utilizando cromatografía, como proponen Cruz et al. (1998), ya que, aunque la columna utilizada es muy eficiente para separar estructuras de ato peso molecular de las de bajo peso, la resolución a alto pesos moleculares no es suficiente como para lograr separarar entre las diferentes PPV que pudieran estar presentes sumultáneamente. En la **Tabla V.6** se muestran las características de diferentes PPV y de rotavirus nativos. Otra estrategia que se podría utilizar sería el tener el mismo número de frascos agitados como de muestras para analizar multímeros. Este acercamiento tiene la desventaja de que limita (por disponibilidad de espacio y de frascos agitados) el número de experimentos que pueden ser conducidos simultáneamente. Además, esta estrategia puede aumentar de forma considerable el error experimental.

Tabla V.6 Características de PPV o partículas de rotavirus nativas.

Multímero	Proteínas[a]	Peso molecular[b] MDa	Diámetro[c] nm
Rotavirus nativo	VP2+VP6+ VP4+VP7	91.78+ARN	81.75
Rotavirus nativo	VP2+VP6	54.72+ARN	70.5
PPV2	VP2+VP6	54.72	70.5
PPV1	VP2	20.4	48

a. Considerando solo las principales proteínas estructurales presentes en los virus nativos. b. Calculados a partir de datos de Mattion et al., 1994. c. Datos de Prasad et al. (1988). Se consideró que las PPV tendrían el mismo diámetro que las partículas obtenidas a partir del baculovirus nativo reportadas por Prasad et al. (1988). Esta suposición es válida ya que micrografías electrónicas han corroborado que las PPV son morfológicamente idénticas que las estructuras del rotavirus nativo (Zeng et al., 1994; Crawford et al., 1994).

En la literatura, el grupo de Labbé et al. (1991) ha utilizado con éxito geles de agarosa para comparar estructuras obtenidas de gradientes de sacarosa. En este trabajo se decidió adaptar esta técnica, que ha sido ya descrita en la **Sección IV.7.6** y en la **Figura IV.3**. La diferencia entre esta técnica y la reportada por Labbé et al. (1991), es que nosotros pretendemos separar los multímeros de extractos celulares completos, mientras que ellos partieron de muestras purificadas con gradientes. Los resultados obtenidos se muestran a continuación. Para confirmar la utilidad de los geles de agarosa para separar las diferentes PPV a partir de extractos celulares completos, multímeros separados por gradientes de CsCl fueron utilizados como estándares. La migración de dichos estándares se muestra en la **Figura V.13A**. Para confirmar que las bandas obtenidas a partir de los extractos celulares fueran producto de las PPV, las proteínas se transfirieron a membranas de nitrocelulosa y se realizó una inmunodetección (**Figura V.13B**). No se observó ninguna banda en los controles de células sin infectar y células recien infectadas. La relación entre la migración de los multímeros y su diámetro y peso molecular (ver **Tabla V.6**) se muestra en la **Figura V.13C**. Es claro que la separación de los multímeros utilizando los geles de agarosa fue eficiente y tuvo una resolución adecuada, aún cuando se utilizaron extractos celulares completos.

Utilizando los geles de agarosa fue posible realizar cinéticas de ensamblaje utilizando muestras de solo 1mL, lo que evitó cambios importantes en el volumen de los cultivos. La principal limitación de esta técnica es que únicamente se pueden separar y cuantificar multímeros en el interior de las células. Las PPV en los sobrenadantes se encuentran en muy baja concentración, por lo que se requieren por lo menos 60 mL de muestra para poder concentrarlos. En algunos experimentos se concentraron los PPV de sobrenadantes al final del cultivo, utilizando una ultracentrifugación a través de un colchón de sacarosa. Sin embargo, fue tanta la manipulación y la cantidad de otros multímeros en el medios de cultivo (por ej. multímeros constituídos por el mismo baculovirus), que fue imposible realizar una cuantificación adecuada

de la concentración de PPV de rotavirus. Por esto, la concentración de PPV en los sobrenadantes únicamente se reporta en forma cualitativa.

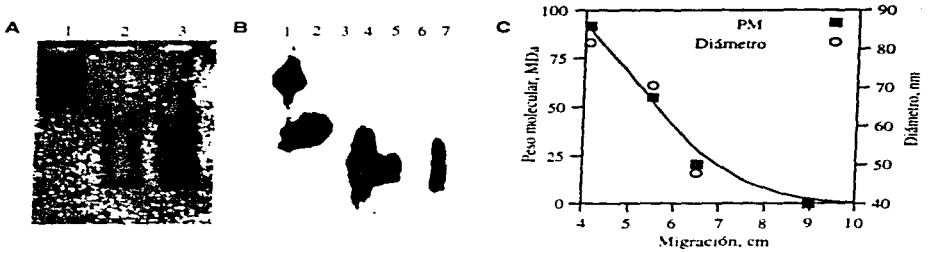

Figura V.13 Separación e identificación de PPVs y rotavirus nativos. A. Gel de agarosa al 0.6%, carril 1: rotavirus completo nativo purificado en gradiente de CsCl, carril 2: rotavirus nativo sin capa externa purificado por gradiente de CsCl, carril 3: PPV2 purificada por gradiente. B. Inmunodetección de gel de agarosa al 0.6%, carril 1, rotavirus completo nativo purificado por gradiente, carril 2: PPV2 purificada por gradiente, carril 3, células no infectadas, carriles 4-6: células infectadas expresando VP2, tiempos postinfección: 48, 24 y 0h, carril 7: PPV1 purificada por gradiente. C. La migración de multímeros en los geles de agarosa graficada contra su diámetro y peso molecular, según datos mostrados en la **Tabla V.6**. Se incluyó albumina bovina (PM= 66 KDa) para comparación. La línea únicamente indica la tendencia.

V.8.1 Producción de PPV1.

La infección de los cultivos únicamente con bac2 lleva a la producción de PPV1. En este caso, y como ya se mencionó al principio de este capítulo, VP2 únicamente se observó en la fracción insoluble de los extractos celulares, en concordancia con otros reportes de la literatura (Labbé et al., 1991, Zeng et al., 1994). En la **Figura V.14** se muestra una representación diagramática de un gradiente obtenido de un extracto celular infectado por bac2 y tratado como se describe en **Materiales y Métodos**, y un gel desnaturalizante de poliacrilamida (8%) teñido con azul de Coomassie de las diferentes fracciones obtenidas. No se observó presencia de VP2 en las fracciones de menor densidad en el gradiente (fracciones 1 y 2 en la **Figura V.14**). Esto indica que aparentemente toda la VP2 presente se ensambló en multímeros, lo que fue observado también por Labbé et al. (1991). Sin embargo, Zeng et al. (1994) han observado el ensamblaje espontáneo de VP2 purificada, por lo que el ensamblaje podría ocurrir fuera de las células en el momento de la purificación. Como se observa en la **Figura V.14**, las fracciones 3, 4 y 5 estaban conformadas por una única proteína que migró a la altura de VP2 (flecha en **Figura V.14**). La identidad de VP2 fue confirmada por inmunodetección (no mostrada). Otros grupos de investigación han observado también la distribución de PPV1 en varias fracciones de

gradientes de sacarosa, y han determinado que esto se debe a la presencia de moléculas de VP2 truncadas, lo que puede evitarse con la adición de inhibidores de proteasas (Zeng et al., 1994, 1996). Sin embargo, en los geles desnaturalizantes no se observó diferencia entre las bandas correspondientes a VP2 de las diferentes fracciones del gradiente (**Figura V.14**). Todas las PPV1 obtenidas por Zeng et al. (1992) de las diferentes fracciones de gradientes de sacarosa fueron morfológicamente idénticas, según observación al microscopio electrónico, a pesar de que algunas contenían las moléculas de VP2 truncadas. Estas moléculas truncadas han sido también observadas en VP2 nativa (Zeng et al., 1994). No se conoce aún el efecto de la presencia de estas moléculas de VP2 incompletas en la calidad de las PPV, ni si la ruptura de VP2 sucede antes o después de su ensamblaje en PPV1.

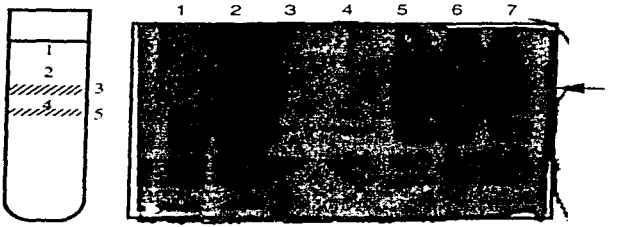

Figura V.14. Purificación de PPV1. Se muestra un diagrama de las bandas obtenidas en el gradiente de CsCl (**ver Materiales y Métodos**). Las regiones sombreadas representan las bandas visibles. Todas las fracciones fueron dializadas y sometidas a electroforesis (gel desnaturalizante de poliacrilamida al 8% teñido con azul de Coomassie). En el gel: Carril 1, extracto celular después de sonicar. Carril 2, paquete celular completo. Carril 3, fracción 1 del gradiente. Carril 4, fracción 2. Carril 5, fracción 3. Carril 6, fracción 4. Carril 7, fracción 5. La flecha señala la banda constituída por VP2, según confirmación por inmunodetección.

Una cinética típica de formación de PPV1 obtenida utilizando geles de agarosa, se muestra en la **Figura V.15**. La cinética de formación de multímeros siguió de cerca a la concentración de VP2, como era de esperarse si toda la proteínas recombinante se ensambla en multímeros. Al final del cultivo, se observó una caída en la concentración de multímeros, que no se observa en la concentración de VP2. Sin embargo, en esta muestra tampoco se observó VP2 sin ensamblar en geles no desnaturalizantes de acrilamida, como los que se describen a continuación. Esta diferencia entre la concentración de proteína y la de PPV1 pudiera por lo tanto deberse a algún evento experimental.

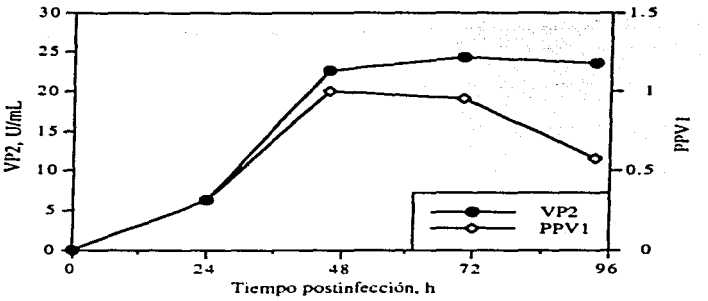

Figura V.15. Cinética típica de producción de multímeros utilizando geles de agarosa. La absorbancia de las bandas en los geles de agarosa fue normalizada con respecto a la de mayor concentración. MDI= 5 ufp/cel.

V.7.2 Producción de PPV2.

Aún cuando se ha reportado la producción de PPV3a (formada por VP6 y VP7) en ausencia de VP2, también se ha reportado que la concentración de estos multímeros es muy baja, y que son muy inestables (Sabara et al., 1991; Crawford et al., 1994; Zeng et al., 1996). En este trabajo no fue posible recuperar PPV de cultivos infectados únicamente con bac6, ni fue posible recuperar PPV4 en ausencia de VP2 (datos no mostrados). En el caso de los cultivos coinfectados con bac2 y bac6, se detectaron bandas que migraron a la altura de los estándares de PPV1 y de PPV2. La cantidad de PPV1 detectada fue más de 100 veces mayor que la de PPV2, según las comparación densitométrica entre las bandas obtenidas (**Figura V.16**). La concentración de PPV1 aumentó conforme avanzó el tiempo después de la infección. PPV2 fue detectada 48 hpi, pero su concentración celular disminuyó después de las 48 hpi. La disminución de la concentración intracelular de PPV2 coincidió con el inicio de la lisis celular, lo que sugiere la liberación de PPV2 al medio de cultivo. Es importante considerar que el análisis de los multímeros se realizó únicamente en los paquetes celulares. Se detectó la presencia de PPV2, pero no de PPV1, en los sobrenadantes de los cultivos coinfectados con bac2 y bac6. Esto coincide con los resultados de Zeng et al. (1996), que al expresar simultáneamente VP2 y VP6 y cosechar al sexto día, únicamente observaron la presencia de PPV2 en el sobrenadante. Aparentemente, para la producción eficiente de PPV2, es necesaria la producción simultánea de VP2 y VP6 por cada célula, para que VP2 forme PPV2 y no permanezca unida a la fracción insoluble de las células. De acuerdo con reportes de la literatura, y el presente trabajo, esta es la única forma en que VP2 puede ser encontrada en la fase celular soluble (Zeng et al., 1996). Por

lo tanto, las estrategias adecuadas para la producción de PPV2 deberán incluir el utilizar MDI mayores de 5 de cada uno de las virus.

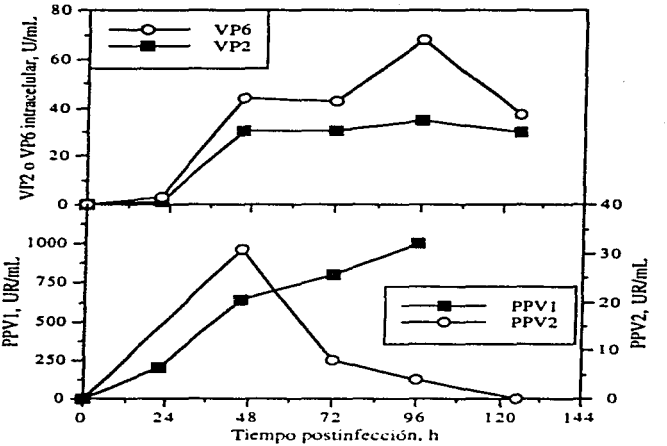

Figura V.16. Cinéticas de VP6, VP2, PPV1 y PPV2 intracelulares en un cultivo infectado simultáneamente con 5 ufp/cel de bac2 y 5 ufp/cel de bac6. UR se refiere a unidades de absorbancia. En los puntos que no se muestran no se detectó ninguna banda en la inmunodetección.

Unicamente el efecto de la ΔTDI sobre la producción de multímeros fue evaluado, y se muestra en la **Figura V.17**. De estos resultados se pueden también sacar conclusiones sobre el efecto de la relación entre las concentraciones de VP6 y VP2 en el ensamblaje de los multímeros. Fue muy interesante observar como, a pesar de que la relación VP6/VP2 disminuyó, la concentración intracelular de PPV2 permaneció prácticamente constante, lo que sugiere un mayor ensamblaje de VP6. Si este es el caso, la disminución de VP6/VP2 trae como consecuencia un mayor aprovechamiento de la VP6 producida al ser ensamblada en multímeros. En el panel inferior de la **Figura V.17**, se observa una disminución en la concentración intracelular de PPV1 conforme disminuye la relación entre VP6 y VP2. Ya que en ninguno de los experimentos se observó una disminución en la concentración de VP2 provocada por el ΔTDI (ver **Figura V.12**), este comportamiento corresponde con el esperado si aumentara la

concentración de VP2 ensamblada en PPV2, y su consecuente liberación al medio de cultivo. No obstante, como se indicó anteriormente, la cuantificación de multímeros extracelulares no fue posible. De igual forma, la disminución en la concentración de PPV1 conforme aumenta la ΔTDI, también corresponde con el comportamiento esperado si PPV2 es liberada al exterioir de las células. Aparentemente, el aumentar el ΔTDI podría aumentar la concentración de proteína ensamblada en PPV2 que estaría en los sobrenadantes de los cultivos. En los 3 cultivos mostrados en la **Figura V.17** se observó PPV2 en el sobrenadante, aunque como ya se mencionó, fue imposible cuantificar su concentración. Este resultados es por demás interesante, aunque mayor experimentación es aún necesaria para tener conclusiones terminantes.

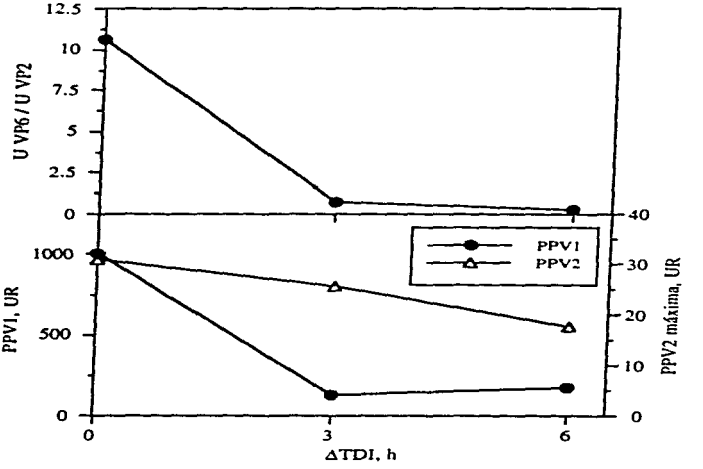

Figura V.17 Efecto del ΔTDI y de la concentración de VP2 y VP6 sobre la concentración intracelular de multímeros. Los experimentos fueron realizados como se explica en la **Sección V.4.2.** UR se refiere a unidades de absorbancia.

Capítulo VI

Conclusiones y Perspectivas

En éste trabajo se abordó de forma integral el proceso de producción de proteínas a través del sistema células de insecto-baculovirus. El estudio incluyó desde los requerimientos nutrimentales de las células, hasta el proponer estrategias computarizadas de control y monitoreo en línea de reactores instrumentados. En cuanto a los requerimientos nutrimentales, se realizó un estudio del metabolismo de las células de insecto antes de la infección, en el que se identificaron las principales fuentes de carbono y energía (Gln y Glc), y el efecto de concentraciones limitantes de éstos nutrimentos. Con éste estudio se logró ampliar el entendimiento de metabolismo celular, y se obtuvo información cuantitativa que puede ser utilizada para el diseño de estrategias de adición de los nutrimentos clave necesarios para mantener la viabilidad de los cultivos y para obtener las máximas concentraciones celulares posibles antes de la infección.

El cultivo en reactores instrumentados es indispensable para la aplicación real del sistema células de insecto-baculovirus. Uno de los problemas más importantes en éste rubro es la fragilidad celular, lo que se ha solucionado utilizando el PF68. Este trabajo se demostró que el PF68 tiene un efecto biológico más allá que el de proteger las células del estrés hidrodinámico. En este renglón se abre un gran abanico de posibilidades de estudio, que incluyen determinar como es la interacción entre las células de insecto y el PF68, y el determinar de forma molecular el efecto de dicha interacción. Este estudio puede tener implicaciones importantes en el uso del PF68 en cultivos de células animales en general, y en el diseño de nuevas sustancias protectoras.

Otro de los retos, y a su vez de las ventajas, de los cultivos en reactores instrumentados, es la factibilidad del diseño de estrategias de monitoreo y control. En éste trabajo, se utilizaron los cultivos en reactores para fortalecer los conocimientos generados en frascos agitados sobre el papel de los diferentes nutrimentos, y su importancia para la producción de proteínas recombinantes. Además, utilizando la VCO se logró determinar en línea la concentración celular, el agotamiento de Gln y Glc, y predecir la productividad de los cultivos infectados con base en el aumento en la VCO después de infectar.

El análisis detallado del proceso de producción de las proteínas multiméricas, ejemplificado con el caso de las PPV de rotavirus, permitió profundizar el entendimiento de los factores

cinéticos que pueden afectar el ensamblaje de multímeros de proteína, como la velocidad de síntesis de cada una de las proteínas recombinantes. En éste trabajo se propusieron y utilizaron dos herramientas para manipular la velocidad de producción de las proteínas. Ambas herramientas permitieron manipular la estequiometría de producción de las proteínas VP2 y VP6 expresadas simultáneamente en células de insecto. Aún es necesario implementar ambas herramientas para el diseño de estrategias de producción de PPV de rotavirus completas, constituidas por las cuatro proteínas. En cuanto a la producción de multímeros, este es el primer trabajo donde se obtienen cinéticas de producción de 2 tipos de PPV simultáneamente, PPV1 y PPV2. Esto se logró gracias a una técnica adaptada de la literatura que requiere pequeñas cantidades de muestra. Las diferentes estquiometrías entre las proteínas recombinantes se vieron reflejadas en diferencias en la concentración y composición de los multímeros obtenidos. Aparentemente, una menor relación VP6/VP2 aumentó la concentración de PPV2, multímero conformado por ambas proteínas.

Además de los cuatro artículos incluidos en ésta Tesis, durante el desarrollo del trabajo doctoral se escribieron dos contribuciones para la Encyclopedia of Cell Technology, las cuales han sido arbitradas y aceptadas para su publicación (Palomares y Ramírez, 1999a; 1999b). Estas contribuciones no han sido incluidas en la Tesis porque no forman parte del proyecto doctoral.

VI.1 El sistema ideal para la producción de PPV2 de rotavirus en células de insecto.

Con éste trabajo se ha logrado incrementar el entendimiento global del sistema de expresión de multímeros de proteínas por células de insecto. Este entendimiento constituye los cimientos del diseño de más y mayores estrategias de producción. Con la información generada en éste trabajo, se pueden proponer las siguientes estrategias para la producción de PPV2 de rotavirus en reactores instrumentados:

1. Utilizar un reactor totalmente instrumentado que permita controlar estrechamente las variables ambientales y monitorear la concentración celular, la concentración de nutrimentos y la infección en general.
2. Agregar PF68 al cultivo, lo que puede incrementar la producción de proteína hasta en 10 veces.
3. Utilizar para ambos virus (bac2 y bac6) una MDI de 5 e infectar utilizando un ΔTDI de 6h. Esta estrategia permitirá utilizar poca cantidad de virus, pero disminuirá la relación VP6/VP2, lo que aparentemente incrementa la producción de PPV2.

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