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Preparation of PLA_2 type IIA Produced by Recombinant *Baculovirus* – Infected Sf21 Insect Cells

E. IONICA[*], E. CONDAC[*], A. DINISCHIOTU[*], M. COSTACHE[*], B. JOHANSEN[**]
[*] *University of Bucharest, Faculty of Biology, 91-95 Spl. Independentei, Bucharest,*
[**] *Norwegian University of Science and Technology, Trondheim, Norway*

Abstract

In this paper we described the production of PLA_2 type IIA into the culture medium of Sf21 insect cells, by using the baculovirus expression system compared to CHO expression system. During the procedure, the necessity for multiplicity of infection (M.O.I.) optimization appeared. When the Sf21 cells were infected with M.O.I. 0.1, the enzyme levels in the culture supernatants reached a maximum of 534 ng/ml on day 4, after that most of the cells were dead. Our results demonstrate that the optimal grade of infection for Sf21 insect cells is only 0.1, suggesting that that type of cells is more sensitive at the virus infection.

Keywords: recombinant PLA_2 type IIA, baculovirus expression system, CHO cells, Sf21 cells,

Introduction

Phospholipase A_2 (PLA_2) enzymes consist of a large family of proteins which share the same enzymatic functions and display considerable sequence homology. At least 15 human genes encode different PLA_2 isoenzymes, including both secreted and cytosolic forms [1]. These enzymes have been characterized in mammalian tissues and snake venoms. PLA_2 catalyzes hydrolysis of the *sn*-2 fatty acyl ester bond of phosphoglycerides, releasing free fatty acids and lysophospholipids. One of the fatty acids that can be released from membrane stores by the activity of PLA_2 is arahidonic acid, the critical precursor for biosynthesis of diverse eicosanoids, including prostaglandins, tromboxanes, and leukotrienes [2].

PLA_2 group IIA is a secreted PLA_2 that has been purified from rheumatoid arthritic synovial fluid [3] and from platelets [4]. The enzyme accumulates at high levels in the joint cavities of patients with rheumatoid arthritis [5,6]. Vadas and his coworkers [5] found that the enzyme purified from synovial fluid caused acute inflammatory and subacute proliferative changes in synovial structures when is injected into knee joint. In addition, PLA_2 group IIA was found to be involved in activation of T lymphocytes with IL-2 [7] and in cardiac ischemia [8]. The enzyme is expressed in human Paneth cells, lacrimal glands, chondrocytes and amniotic epithelial cells [9,10,11,12]. The PLA_2 group IIA gene (*PLA2G2A*) seems to play diverse roles in human diseases, including colon cancer, coronary artery disease, and inflammation [13,14,15]. Induction of *PLA2G2A* is a frequent feature of inflammatory responses. Elevated expression of *PLA2G2A* has been reported in several types of malignancies, including pancreatic cancer and prostate cancer [16,17].

To study the multitudes of PLA_2 type IIA functions, a large amount of enzyme is necessary for *in vitro* and *in vivo* experiments. In the late '80, PLA_2 type IIA was purified

from blood and synovial fluid of patients with rheumatoid arthritis. The PLA2G2A gene was cloned by using the information from GENBANK. The whole gene occurred within a 3.8 kb fragment was integrated in simian virus 40 (SV40) at the level of *Eag*I/*Hin*d3 restriction site [18,19]. The obtained vector was transfected in African Green monkey kidney (COS) cells in order to obtain a transitory expression of PLA_2 type IIA. The enzyme was secreted into the medium and a concentration of 3 ng/ml was observed.

Other research groups demonstrated that more quantities of pure enzyme are necessary. PLA_2 type IIA was cloned and expressed in CHO mammalian cells [18] and Sf9 insect cells [20], but the production of recombinant PLA_2 was not much higher.

It has been shown that baculovirus expression system using *Ac*NPV (*Autographa californica* nuclear polyhedrosis virus) in Sf cells expresses a wide variety of eukaryotic genes, often producing the proteins at much higher levels that other expression vector systems.

In this paper we describe the production of PLA_2 type IIA into the culture medium of Sf21 insect cells, by using the *Baculovirus* expression system compared with CHO expression system [18].

Methods

Expression of PLA_2 type IIA in CHO (II-F7) mammaliam cells

The plasmids pBG341:::3.8 and pAdD26-1, were used to construct the mammalian expression vector for PLA_2 type IIA. Both plasmids were the products of Kramer [17] and Schahill [21] laboratories. CHO cells deficient in dihydrofolate reductase (*dhfr*) gene (CHO^-) were maintained at 37^0C in α-MEM (GIBCO) medium supplemented with 10% fetal bovine serum. The expression vector used for transfection experiments, pBG341:::3.8 contained a 3,8 kb fragment which include the gene for PLA_2 type IIA and use the adenovirus-2 major late promoter (AMLP) with the SV40 enhancer [22]. The wild gene for dihydrofolate reductase is included in pAdD26-1 plasmid and is used for selecting cells that incorporated plasmid DNA, and then for amplifying the gene. This was obtained by co-transfection of 1×10^6 CHO cells ($dhfr^-$) with 20 μg DNA from pBG341:::3.8 and 2 μg DNA from pAdD26-1 ($dhfr^+$). The transfection was achieved by the $Ca_3(PO_4)_2$ precipitation method [17]. After 7 days of cultivation the CHO^+ ($dhfr^+$) cells were selected for growth in α-MEM containing 30 nM methotrexate (MTX). The clones obtained by serial dilution were isolated and cultivated separate to obtain the enzyme.

Expression of PLA_2 type IIA in Sf21 insect cells

The baculovirus expression system (SF21 insect cells culture and CLONTECH BacPAK6) was adapted in order to obtained large quantities of PLA_2 type IIA. The baculovirus system offers some advantages compared with prokaryotic, yeast, and mammalian expression systems. In most cases the post-translational processing of foreign eukaryotic proteins expressed in insect cells is similar enough to that found in mammalian cells. The produced protein have a comparable biological activity. The baculovirus system is a specially engineered virus which facilitates the construction and selection of recombinant expression vectors. BacPAK6 use an essential gene adjacent to the polyedrin locus which provide a selection for recombinant virus [23]. Into the gene, flanking the polyedrin expression locus of BacPAK6, sites for *Bsu*36I were introduced. Digestion of BacPAK6 viral DNA with *Bsu*36I releases two small fragments, one of which is carrier part of the downstream gene, ORF1629, essential for viral replication.

Insect cell culture

The insect cell line IPLB-Sf21 (Sf21) was supplied with the BakPAK6 kit, for propagation of *Ac*NPV expression vector. The culture of insect SF21 cells and virus production were performed as described by CLONTECH Laboratories Manual [24]. A vial of IPLB-Sf21 cells containing $2x10^6$ cells in 1 ml of TNM-FH (Grace's insect cell medium)/10% FBS/10% DMSO was introduced into 25 cm^2 flask and warmed to 37^0C waterbath with gentle agitation until the cell suspension is almost thawed. In a laminar flow hood, the cell suspension is transfer into a 25 cm^2 flask which contain 5 ml TNM-FH medium at 27^0C, and incubate for 1-3 hours. After, a significant fraction of cells was attached, the medium was gently removed and replaced with a fresh aliquot (5 ml) of prewarmed TNM-FH/FBS medium. Incubate at 27^0C until the cells have formed a near-confluent monolayer (approximate 7 days). The subculturing of Sf21 monolayer was realized in 150 cm^2 flasks and after that the suspension cultures were transferred in shake flasks (500 ml). The suspension cultures are particularly useful when large numbers of cells are needed.

Construction of recombinant transfer vector pAcYM1-PLA_2

The *PLA2G2A* DNA fragment of 460 bp, purchased from Kramer laboratories was digested with *Bgl*II and inserted at the *Bam*HI site of the mp18 phage vector (**Figure 1**). The PLA_2-DNA was obtained by cleaving mp18-PLA_2 with *Xho*II. The insertion of *PLA2G2A* DNA was achieved at the *Bam*HI site of p*Ac*YM1. The transfer vector p*Ac*YM1-PLA_2 was used to prepare the recombinant vector.

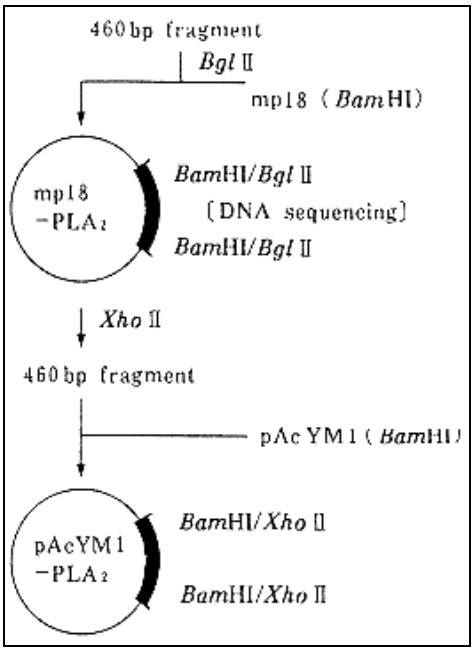

Figure 1. Construction of recombinant transfer vector p*Ac*YM1-PLA_2

Preparation of the recombinant virus

The recombinant transfer vector p*Ac*YM1-PLA_2 and the fragment of BacPAK6 viral DNA digested with *Bsu*36I were cotransfected into $1x10^6$ Sf21 cells. The cotransfection was done with 50µl lipofectin (diluted 1:50) and 50µl mixture (100 ng/ml *Ac*-PLA_2 DNA, viral DNA digested with *Bsu*36I, sterile water). After 15 min. the DNA-lipofectin complex was added to the medium and incubated at 27^0C for 5 hours. 60-72 hours after addition of lipofectin-DNA complexes to the cells, the medium which contain viruses produced by the transfected cells was transferred to a sterile container and stored at 4^0C.

Production of PLA_2 type IIA by Ac-PLA_2-infected Sf21 cells

To obtained more quantities of PLA_2 type IIA proteins, we used 100-500 ml suspension culture with $2x10^5$ Sf21 cells/ml, and infected them by adding the requisite volume of virus stock until reach $1x10^6$ cells/ml. To achieve maximal protein expression, a high-quality medium was used, with fetal bovine serum and Sf21 cells in log phase, which are at least 98% viable.

The cells were cultured in 200 cm^2 flasks and infected with the recombinant *Ac*-PLA_2 virus for 7 days at 27^0 C. The infection was realized by adding virus at different M.O.I. (multiplicity of infection): 10µl for M.O.I. of 0.1; 100µl for M.O.I. of 1.0 and 1000µl for M.O.I. of 10. 500 µl aliquots were picked up in each day. The cells were pelleted in a microcentrifuge at low speed (1.000 r.p.m.) for 1 min and the supernatant was stored at -20^0C. The samples were analyzed in every day of infection for PLA_2 type IIA activity by using a NEFA – C kit.

Measurement of PLA_2 type IIA activity

PLA_2 type IIA activity was determined in the samples with a NEFA-C kit (Wako Chemical GmBH) according to the following procedure (**Figure 2**). A substrate solution was prepared by dissolving 10 mg L-α-phosphatidylcholine-β-oleoyl-γ-palmitoil (Sigma) in 200 µl 4% Nonidet P40 and 2% deoxycholic acid (sodium salt) with vortex. 1.8 ml of 0.12 M Tris-HCl pH 8.0 containing 12 mM $CaCl_2$, and 0.1 mM EDTA was added and the solution was thorough votexed.

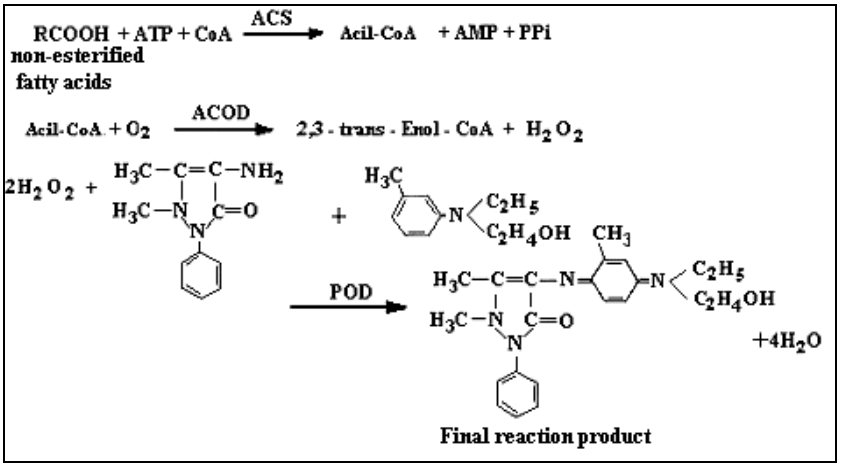

Figure 2. Chemical principles of NEFA-C kit (according to the procedure of Wako Chemical GmBH). ACS- acyl coenzyme A synthetase, AOD- ascorbate oxidase, CoA- coenzyme A, POD- peroxidase

Before use, the substrate solution was incubated at 37^0C for 30-60 min. A 96 well plate was blocked with 10 mg/ml gelatin in PBS for 4 hours at RT (room temperature). 10 µl sample was transferred to each well and the plate was preheated at 37^0C for 3 min. 20 µl substrate solution was added and the plate incubated at 37^0C for 20 min. 80 µl of reagent A from NEFA-C kit (prepared according to the manufacturers' procedure) was added and the plate incubated at 37^0C for 10 min. Finally, 150 µl of reagent B from NEFA-C kit (prepared according to the manufacturers' procedure) was added and the absorbance was measured at 550 nm after 5-10 min at RT. To correct for background levels and non-enzymatically derived free fatty acids, wells were included with sample but only substrate solution.

Results and Discussions

CHO-II-F7 – pBG341:::3.8 system described by us was used as a control of PLA_2 type IIA protein production in mammalian cells. The expression vector used for transfection experiments contain one of the adenovirus-2 major later promoter (AMPL) coupled with the SV40 enhancer [22]. The cotransfection of mammalian cells with pBG341:::3.8 vector and the plasmid that contains the *dhfr* gene allow us to obtained a stable CHO cell line. Activity measurements of stable cell lines after amplification indicated that the levels of PLA_2 type IIA activity had increased 2-3 fold over levels observed in the parental lines. Using this system, as a control, we optimized the culture and infection procedures for Sf21 insect cells.

The PLA_2 type IIA DNA and recombinant transfer vector p*Ac*YM1-PLA_2 was constructed as described previously. The resulting recombinant transfer vector had the PLA_2 type IIA sequence including the signal peptide sequence and the 5' regulatory region of the polyedrin gene.

Ac-PLA_2 and the AcNPV DNA (BacPAK6) were cotransfected in Sf21 cells by using the calcium phosphate method. A few of the about thousand plaques were selected for presence of the recombinant virus which was purified by plaquing twice and then propagated in the Sf21 cells. In the end we obtained a recombinant baculovirus, *Ac*-PLA_2, which carry the *PLA2G2A* gene under the control of the polyedrin promoter.

At this moment of the experiment the necessity of multiplicity of infection (M.O.I.) optimization appeared. The values which were obtained at different levels of PLA_2 type IIA activity by modifying the period of infection and the multiplicity, are presented in (**Table 1**).

To calculate the activity of PLA_2 type IIA which was secreted into the Sf21 medium, after virus infection, we have considered only the values that are included in the linear range of PLA_2 type IIA etalon curve (**Figure 3**). The results are shown in (**Table 2, Figure 4**).

Table 1. PLA_2 type IIA activity at different period of infection and M.O.I. (MOI – multiplicity of infection, OD – absorbance, FD – dilution factor)

MOI	Period of infection (days)	OD550 (nm)	OD550 (nm)	ng/ml $sPLA_2$x FD (10)	OD550 (nm)	ng/ml $sPLA_2$ x FD (50)
0.1	0	0.127	0.199	69	0.177	235
	1	0.968	**0.193**	**63**	0.131	5
	2	1.084	**0.36**	**230**	0.189	295
	3	1.03	**0.664**	**534**	0.239	545
	4	1.052	**0.403**	273	0.214	420
	5	1.064	0.503	373	**0.206**	**380**
	6	1.01	0.552	422	**0.22**	**273**
	7	1.077	0.48	350	**0.182**	**250**
1	0	0.133	0.12	0	0.12	0
	1	1.072	0.433	303	0.189	295
	2	1.103	**0.464**	**334**	0.19	300
	3	1.101	**0.429**	**299**	0.199	345
	4	1.132	**0.432**	**302**	0.164	170
	5	1.108	**0.33**	**200**	0.161	155
	6	1.085	**0.322**	**192**	0.157	135
	7	1.13	**0.232**	**102**	0.168	190
10	0	0.13	0.125	0	0.118	0
	1	1.032	0.563	433	**0.213**	**415**
	2	1.096	0.531	401	**0.204**	**370**
	3	1.088	0.502	372	**0.192**	**310**
	4	1.104	0.447	317	**0.19**	**300**
	5	1.055	0.465	335	**0.18**	**250**
	6	1.1	**0.413**	**283**	0.173	215
	7	1,029	**0.499**	**369**	0.198	340

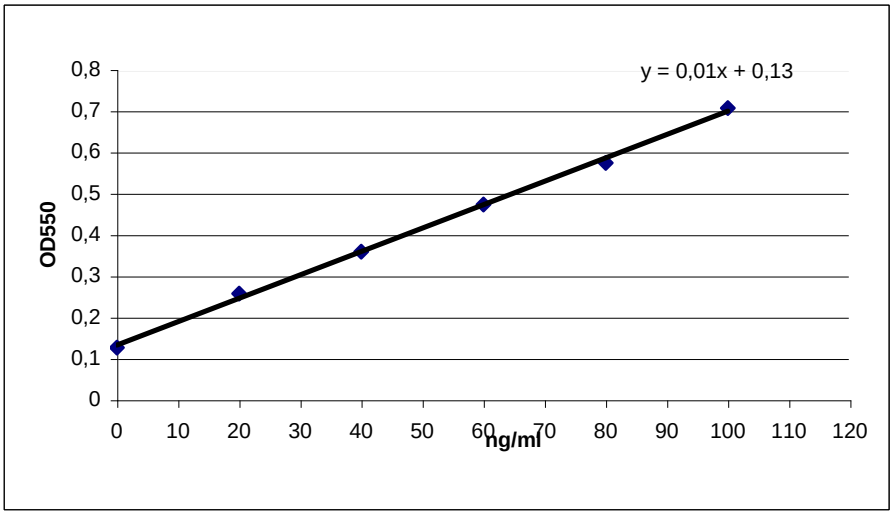

Figure 3. PLA_2 type IIA activity: etalon curve

When the Sf21 cells were infected with M.O.I. 0.1, the enzyme levels in the culture supernatants reached a maximum of 534 ng/ml on day 4, after that most of the cells were dead. When the cells were infected with an M.O.I. =1, the enzyme was secreted until the 2rd day, and the maximum concentration was 334 ng/ml. After that, the concentration is maintained in the range of 200 ng/ml. When M.O.I. is 10, the concentration of recombinant virus that infected the cells was too high and the cells were killed from the first day.

Our data indicate that PLA_2 type IIA production in the current baculovirus expression system was three orders higher than those achieved in mammalian cells expression systems. This would be due to the fact that the PLA_2 type IIA gene was constructed using the polyedrinc promoter and the codons which appear usual in polyedrinic gene.

Other groups were recommended a M.O.I. range between 5 and 25, in order to obtain sufficient protein for purification. Our results demonstrate that the optimal grade of infection for Sf21 insect cells is only 0.1, suggesting that that type of cells is more sensitive at the virus infection.

Table 3. Variation of PLA_2 type IIA activity secreted into the Sf21 medium, after virus infection

Period of infection	PLA_2 type IIA (ng/ml)		
	MOI = 0,1	MOI = 1	MOI = 10
0	0	0	0
1	63	303	415
2	230	334	370
3	534	299	310
4	403	302	300
5	380	200	250
6	273	192	283
7	250	102	369

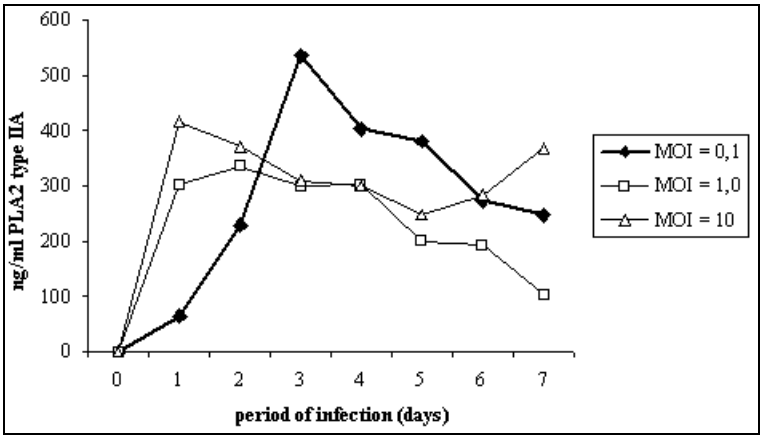

Figure 4. Variation of PLA_2 type IIA activity secreted into the Sf21 medium with period of infection

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