Journal of Industrial Microbiology & Biotechnology (1998) 21, 175–177

http://www.stockton-press.co.uk/jim

Expression of human PAI-2 in the baculovirus expression system

X-W Zhang[1], T Sun[1], X-F Zhou[2], X-Y Zeng[1], X Liu[1] and D-X Gu[1]

[1]*Food Engineering Research Center of State Education Commission, Zhongshan University, Guangzhou;* [2]*The Occupational Disease Prevention and Control Hospital of Guangdong Province, Guangzhou, People's Republic of China*

Using pSXIVVI$^+$X3 as an expressing vector, an occluded recombinant *Trichoplusia ni* nuclear polyhedrosis virus carrying the cDNA encoding plasminogen activators inhibitor-2 (PAI-2) under the control of the Syn and XIV promoters, has been constructed. SDS-PAGE and immunoblot analysis revealed that the virus-mediated PAI-2, with a molecular weight of ~45 kDa, was synthesized in the Sf cells at a level of ~16% of total intracellular protein and in the supernatant phase at a level of ~64% of total extracellular protein secreted into the hemolymph of infected larvae. The expressed protein was similar to its authentic counterpart in terms of immunoreactivity and bioactivity.

Keywords: plasminogen activator inhibitor-2; baculovirus; expression vector; secretion

Introduction

Plasminogen activators (PAs) are enzymes that convert the inactive zymogen plasminogen into the trypsin-like protease plasmin, which degrades fibrin as well as fibronectin and other extracellular matrix proteins [4,12]. PAs play an important role in fibrinolysis [3], inflammation [16], invasive and metastatic growth [4,8], morphogenetic remodeling and other forms of cell migration [15]. The activity of PAs is controlled at multiple levels, including the regulated synthesis of PA-specific inhibitors (PAIs). One of these PAIs, originally purified from human placental extracts [19,21] and now called PAI-2, is found in a nonglycosylated intracellular and a glycosylated secreted form without the cleavage of a signal peptide [2,5].

The baculovirus expression vector system has been widely used to express a variety of heterologous genes in insect cells and caterpillars [7,10,11]. Insect cells provide a suitable environment for post-translational modifications and folding of the protein product such that the foreign proteins synthesized resemble their authentic counterparts in almost all respects [7], hence this remains the system of choice for expressing genes for therapeutic or vaccine purposes.

In this communication, because most of the recombinant PAI-2 expressed in *E. coli* and mammalian cells was in the form of inclusion body and inconvenient for purification [9,13,20], a cDNA coding for human plasminogen activator inhibitor-2 (PAI-2) was inserted into a transfer vector plasmid pSXIVVI$^+$X3, an occluded recombinant TnNPV carrying the cDNA encoding human PAI-2 under the control of the Syn and XIV promoters has been constructed. The expressions of PAI-2 protein in *Spodoptera frugiperda* (Sf9) cells and *Plusia agnata* larvae were investigated.

Correspondence: X-W Zhang, Food Engineering Research Center of State Education Commission, Zhongshan University, Guangzhou, People's Republic of China

Abbreviations: FBS, fetal bovine serium; IgG, immunoglobulin; kb, kilobase(s); MOI, multiplicity of infection; PAGE, polyacrylamide gel electrophoresis; PAs, plasminogen activators; PAI-2, plasminogen activator inhibitor-2; PBS, phosphate-buffered saline; p.i., postinfection; PMA, phorbol myristate acetate; Polh, polyhedrin promoter; re-BV, recombinant baculovirus; SDS, sodium dodecyl sulfate; Sf9, *Spodoptera frugiperda* clone 9 cells; Syn, synthesis promoter; TnNPV, *Trichoplusia ni* nuclear polyhehrin virus; XIV, XIV promoter.

Received 5 May 1998; accepted 15 July 1998

Materials and methods

Construction of transfer vector carrying PAI2 cDNA

The *Eco*RI fragment carrying the cDNA encoding PAI-2 from PAI-2/pEM (Dr Xiang-Rong Cao, Nanjing Normal University, Nanjing, China) was cloned into the *Eco*RI site of transfer vector plasmid pSXIVVI$^+$X3 (5.8 kb) [17,18]. The ligation mix was transformed in *E. coli* DH5, thus replasmid pSXIVVI$^+$X3-PAI2 (7.7 kb) was generated, and the correct orientation for PAI-2 cDNA inserted was confirmed by detailed analysis (data not shown).

Western blot analysis

Four instars of *Plusia agnata* larvae were fed with diet coated with recombinant virus polyhedrin, six instars of larvae were harvested 4 days post-infection. Larvae were bled by cutting off the prolegs, hemolymph was collected, centrifuged and filtered, and the supernatant was used for further analysis. SDS-PAGE was performed using slab gels with 10% polyacrylamide separating gel and 4% stacking gel. Western blot analysis was carried out as described [5]. Western blots of larval proteins separated by SDS-PAGE were probed by rabbit anti-human PAI-2 polyclonal antibody [21] at a dilution of 1 : 1000; the membranes were incubated in goat anti-rabbit IgG conjugated with horseradish peroxidase (BioRad Laboratories, Richmond, CA, USA) used at a dilution of 1 : 400 according to the manufacturer's directions.

Activity assay

Recombinant virus solutions (1.5 ml) were infected with Sf9 cells (3×10^6 cell, MOI = 10 pfu cell^{-1}); after 1 h the virus solutions were dislodged and 3 ml of medium Tc-100 containing 10% FBS was added. Cell samples and super-

natant medium were harvested after 24 h postinfection. PAI-2 activity was measured by the fibrin plate technique [1,6].

Results and discussion

Construction and characterization of re-BV containing PAI-2 gene

The partial physical map of the transfer vector, pSXIVVI$^+$X3-PAI-2, used for generating recombinant virus, is illustrated in Figure 1. For constructing the re-BV, Sf9 insect cells (NERC, Institute of Virology, Oxford, UK) were co-transfected with the replasmid and TnNPV-SVI$^-$G DNA, a recombinant virus (gal$^+$OCC$^-$) [17,18]. The re-BV designated TnNPV-PAI-2-OCC$^+$, which contains PAI-2 cDNA and can form polyhehrin, was obtained by plaque purification [11,14]. Enzyme digestion identification revealed that *Eco*RI+*Sac*I digested re-BV has a 1.9-kb band, which is not visible in the two-enzymes-digestion map of parent virus (data not shown).

Expression of PAI-2 cDNA in insect cells and larvae

Sf9 cells were infected with re-BV TnNPV-PAI-2-OCC$^+$. SDS-PAGE and Western blot analysis reveal that PAI-2 can be detected in the infected cell culture supernatant and cells 24 h p.i., and the following mathematical equations can be used to fit the relationship between the activity of PAI-2 ($A, \times 10^3$ u ml^{-1}) and the time after infection (t,h):

$$\text{for cells } A = -0.0006\, t^2 + 0.1220\, t - 2.5080 \quad (1)$$

$$\text{for medium } A = -0.0010\, t^2 + 0.1949\, t - 3.9860 \quad (2)$$

According to Eqns (1)–(2), the activity of PAI-2 reaches its maximum value 3.6937 and 5.5065 ($\times 10^3$ u ml^{-1}) at 101.6 h and 99.5 h after infection for cells and medium, respectively. The fitting results are satisfactory (Figure 2). Densitometric scanning of SDS-PAGE revealed that ~16% of the total Coomassie blue-stainable protein of the infected cells was represented by the recombinant PAI-2, and ~64% of the total extracellular protein was the secreted PAI-2 protein (data not shown). This is in agreement with the fact that ~70% of PAI-2 was secreted into the medium by PMA-stimulated U937 cells [21], although PMA stimulation is not needed in the present infected cell.

P. agnata larvae were infected with the re-BV TnNPV-PAI-2-OCC$^+$ simply by mixing the re-BV into the insect

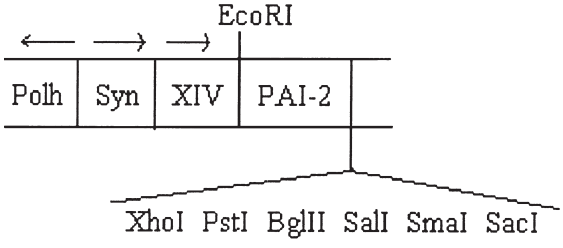

Figure 1 Partial physical map of the transfer vector pSXIVVI$^+$X3-PAI-2 showing the composition of the promoter cassettes and the direction of transcription of the genes encoding PAI-2. Relevant restriction sites, used in checking the orientation of the insert with respect to the promoter, are indicated. Polh, polyhedrin promoter; Syn, synthesis promoter; XIV, XIV promoter.

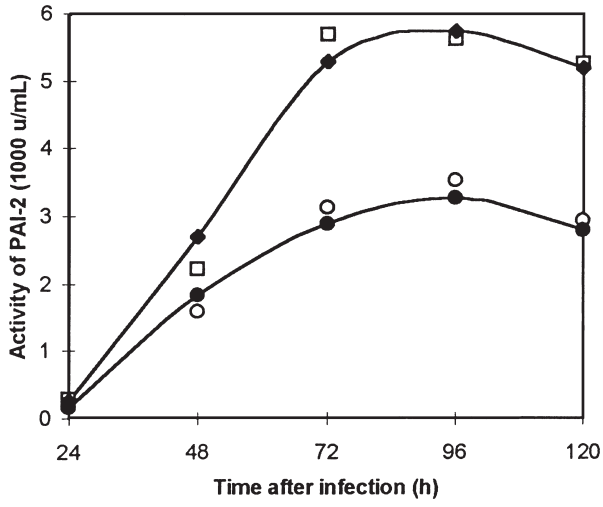

Figure 2 Time course of re-BV expressing PAI-2 in Sf9 cells and supernatant. (○) Experimental for cell; (●) calculated for cell; (□) experimental for medium; (◆) calculated for medium.

diet. Hemolymph was taken, centrifuged and filtered. The supernatant was analyzed by SDS-PAGE and Western blot; the results show that PAI-2 can also be expressed in the larvae, with a molecular weight of ~45 kD, reacted with rabbit anti-human PAI-2 polyclonal antibody, and secreted into hemolymph (Figure 3). This proves that the recombinant PAI-2 is similar to the native.

Recently, Zhou *et al* [22] developed a high-level expression system for PAI-2 by inserting a modified PAI-2 gene downstream of the T7 promoter, the expression level of recombinant PAI-2 amounted to about 60% of the total cellular proteins, and most of the recombinant PAI-2 expressed in *E. coli* was in the form of inclusion body. While in this report, although the expressed recombinant PAI-2 amounted to about 16% of total intracellular protein, the majority of this recombinant protein was secreted into

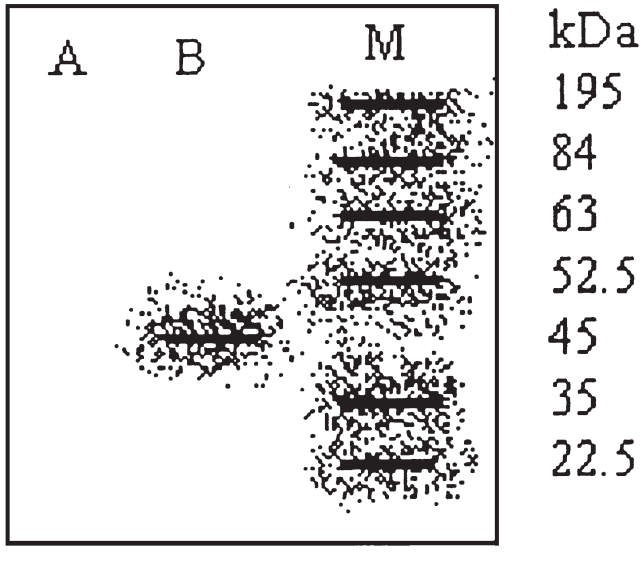

Figure 3 Western blot of PAI-2 expression using re-BV in larvae. (A) Larvae infected with TnNPV for 96 h p.i. (B) Larvae infected with re-BV TnNPV-PAI-2-OCC$^+$. (M) Molecular weight markers.

the medium (about 64% of total extracellullar protein), which was convenient for purification. The recombinant proteins can be produced in caterpillars, which is valuable for the inexpensive mass-scale production of such proteins.

Conclusions

(1) The cDNA encoding human PAI-2 can be expressed successfully in an occluded recombinant baculovirus expression system with the control of synthetic and XIV promoters. This new recombinant baculovirus can infect larvae simply by mixing them into the insect diet.
(2) About 64% of recombinant PAI-2 was secreted into the medium, while about 16% of PAI-2 was retained in the cell. The expression of recombinant PAI-2 reaches a maximum value after 4 days post-infection.
(3) Biological and immunological properties clearly indicate that the virus-mediated PAI-2 is of potential utility in biochemical, immunodiagnostic and clinical applications.

References

1 Astrup T and S Mullertz 1952. The fibrin plate method for estimating fibrinolytic activity. Arch Biochem Biophys 40: 346–351.
2 Belin D, A Wohlwend, WD Schleuning, EKO Kruithof and JD Vassalli. 1989. Facultative polypeptide translocation allows a single mRNA to encode the secreted and cytosolic forms of plasminogen activators inhibitor 2. EMBO J 3287–3294.
3 Collen D. 1980. On the regulation and control of fibrinolysis. Thromb Haemostasis 43: 77–89.
4 Danϕ K, PA Andreasen, J Grϕndahl-Hansen, P Kristensen, LS Nielsen and L Skriver. 1985. Plasminogen activators, tissue degradation and cancer. Adv Cancer Res 44: 139–266.
5 Genton C, EKO Kruithof and WD Schleuning. 1987. Phorbol ester induces the biosynthesis of glycosylated and nonglycosylated plasminogen activator inhibitor 2 in high excess over urokinase-type plasminogen activator in human U-937 lymphoma cells. J Cell Biol 104: 705–712.
6 Kruithof EKO, JD Vassalli and WD Schleuning. 1986. Purification and characterization of a plasminogen activator inhibitor from the histiocytic lymphoma cell line U-937. J Biol Chem 261: 11207–11213.
7 Luckow VA and MD Summers. 1988. Trends in the development of baculovirus vectors. Biotechnology 6: 45–55.
8 Markus G. 1984. The role of haemostasis and fibronolysis in the metastatic spread of cancer. Semin Thromb Haemostasis 10: 61–70.
9 Mikus P, T Urano, P Liljestrom and T Ny. 1993. Plasminogen activator inhibitor type 2 is a spontaneously polymerising SERPIN. Eur J Biochem 218: 1071–1082.
10 Miller LK. 1988. Baculovirus as gene expression vectors. Annu Rev Microbiol 42: 177–199.
11 O'Reilly DR, LK Miller and VA Luckow. 1992. Baculovirus Expression Vectors—A Laboratory Manual. WH Freeman and Company, New York.
12 Sksela O. 1985. Plasminogen activator and regulation of pericellular proteolysis. Biochem Biophys Acta 823: 35–65.
13 Steven J, IR Cottingham, SJ Berry, SA Chinery, AR Goddey, M Courtney and DJ Ballance. 1991. Purification and characterization of plasminogen activator inhibitor type 2 produced in *Saccharomyces cerevisiae*. Eur J Biochem 196: 431–438.
14 Summers MD and GE Smith. 1987. A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures. Texas Agric Exp Stn Bull 1555, College Station, TX, USA.
15 Valinsky JE, E Reich and NM Le Douarin. 1981. Plasminogen activator in the bursa of *Fabricius*: correlations with morphogenetic remodeling and cell migrations. Cell 25: 471–476.
16 Vassalli JD, J Hamilton and E Reich. 1976. Macrophage plasminogen activator: modulation of enzyme production by antiinflammatory steroids, mitotic inhibitors, and cyclic nucleotides. Cell 8: 271–281.
17 Wang X, BG Ooi and LK Miller. 1991. Baculovirus vectors for multiple gene expression and for occluded virus production. Gene 100: 131–137.
18 Wang X, W Xie, Q Long, D He, G Lin and Y Pang. 1992. Stimulation of thymidine kinase activity in baculovirus infected cells is not due to virus-coded enzyme. Arch Virol 127: 315–319.
19 Wun TC and E Reich. 1987. An inhibitor of plasminogen activation from human placenta. J Biol Chem 262: 3646–3653.
20 Ye RD, TC Wun and JE Sadler. 1987. cDNA cloning and expression in *E. coli* of plasminogen activator inhibitor from human placenta. J Biol Chem 262: 3718–3725.
21 Ye RD, TC Wun and JE Sadler. 1988. Mammalian protein secretion without signal peptide removal. J Biol Chem 263: 4869–4875.
22 Zhou A, X Jiang, F Dou, D Zhu and X Xu. 1997. Renaturation, purification, and characterization of human plasminogen activator inhibitor type 2 (PAI-2) accumulated at high level in *Escherichia coli*. J Biochem 121: 930–934.