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CICLO XXII

TITOLO DELLA TESI DI DOTTORATO

Pichia pastoris:
AN EUKARIOTIC SYSTEM FOR THE EXPRESSION OF RECOMBINANT THERAPEUTIC MOLECULES

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ABSTRACT

Rationally designed anticancer agents targeting cell-surface antigens or receptors, such as Immunotoxins, represent a promising approach for treating cancer patients. The Immunotoxin IL4PE40 derives its activity to induce apoptosis from the 40 kDa PE fragment (*Pseudomonas* exotoxin A) and its specificity from the permuted form of interleukin-4. IL4PE40 produced in *E. coli* was extracted from inclusion bodies and purified by affinity chromatography, in its active form. However, the clinical translation of therapeutic molecules produced in *E. coli* is often difficult, due to endotoxin contaminations and laborious protocols of inclusion bodies purification that make hard the reproducibility of the isolation procedures. To overcome these disadvantages, I cloned the IL4PE40 gene into a yeast expression vector for use in the *Pichia pastoris* yeast. In the literature it is reported that *P. pastoris* can secrete grams per liter of foreign proteins in a protein-poor medium that is inexpensive and chemically defined. The presence of a signal peptide at the N-terminus of the recombinant protein allows the secretion of native protein within the culture medium. In addition, the low level of endogenous proteins secreted in the culture medium facilitates the purification steps of the final product. In my work the yield of IL4PE40 produced by yeast was optimized by yeast codon usage and by testing different induction conditions. After optimization the yeast *P. pastoris* was able to produce 120 µg of full-length Immunotoxin from 1 liter of culture, which is far from results obtained with non-toxic proteins by other workers. Moreover, although after optimization the yield increased, proteins obtained from optimized cultures of *P. pastoris* show a considerable level of degradation. The translocation of toxic fusion proteins in the cytosol during biosynthesis may lead to such low protein production. Additionally, it appears that proteases cleaving the recombinant molecules cold be present in the yeast medium. Preliminary cleavage assays with native PE and literature data suggest that several proteases (serine proteases and subtilisins) could cooperate in the IL4PE40 cleavage. Planned experiments to overcome these limitations are also described in my thesis.

RIASSUNTO

L'ideazione di farmaci antitumorali che riconoscono recettori o antigeni della superficie cellulare, come le Immunotossine, rappresentano un approccio promettente per il trattamento di pazienti affetti da cancro. La capacità di indurre l'apoptosi dell'Immunotossina IL4PE40 deriva dal frammento di 40 kDa della tossina PE (*Pseudomonas* esotossina A) e la sua specificità dalla forma permutata dell'interleuchina-4. IL4PE40 prodotta da *E. coli* è estratta dai corpi d'inclusione e purificata nella sua forma attiva tramite cromatografia di affinità. Comunque, la translazione clinica di molecole terapeutiche prodotte da *E. coli* è spesso difficoltosa, a causa di contaminazioni da endotossine e di protocolli laboriosi per la purificazione dei corpi d'inclusione che rendono difficile la riproducibilità della procedura di isolamento. Per superare questi svantaggi, ho clonato il gene di IL4PE40 in un vettore d'espressione per il lievito *Pastoris pastoris*. In letteratura è riportato che *P. pastoris* è in grado di secernere grammi per litro di proteine eterologhe in un terreno povero di proteine, economico e chimicamente definito. La presenza di un peptide segnale all'N-terminale della proteina ricombinante permette la secrezione della proteina nativa nel terreno di coltura. Inoltre il basso livello di secrezione di proteine endogene nel terreno di coltura, facilita i passaggi di purificazione del prodotto finale. Nel mio lavoro la resa di IL4PE40 prodotta dal lievito è stata ottimizzata dal utilizzo del "codon usage" del lievito e dall'analisi di diverse condizioni di induzione. Dopo l'ottimizzazione *P. pastoris* è in grado di produrre 120 µg di Immunotossina da un litro di coltura; questo quantità è lontana dai risultati ottenuti in altri lavori con proteine non tossiche. Inoltre, benché dopo l'ottimizzazione la resa sia aumentata, le proteine ottenute da colture ottimizzate di *P. pastoris* mostrano un considerevole livello di degradazione. La translocazione della proteina tossica nel citosol durante la biosintesi potrebbe portare ad una dminuzione della produzione. Inoltre, sembra che nel terreno del lievito siano presenti proteasi in grado di degradare la molecola ricombinante. Saggi di degradazione con la PE nativa e dati della letteratura suggeriscono che diverse proteasi (serine e subtilisine) potrebbero cooperare nella degradazione di IL4PE40. Nella mia tesi sono inoltre descritti esperimenti pianificati per superare queste limitazioni.

INDEX

ABBREVIATIONS

IPTG: isopropyl-β-D-thiogalactoside.

h: hours

min: minutes

PBS: phosphate-buffered saline

Sec: seconds

r.t.: room temperature

o.n..: over night

Fv: Variable fragment

scFv: single chain variable fragment

mAb: monoclonal antibody

CLL: chronic lymphocytic leukaemia

VLS: Vascular leak syndrome

PE: *Pseudomonas* exotoxin A

PE38: 38 kDa fragment of *Pseudomonas* exotoxin A

PE40: 40 kDa fragment of *Pseudomonas* exotoxin A

IL-4: interleukin-4

CP-IL4: circularly permuted interleukin-4

EF-2: Elongation factor-2

DT: diphtheria toxin

RIP: ribosome inactivating protein

1. INTRODUCTION

1.1 PRODUCTION OF RECOMBINANT PROTEINS: HISTORICAL OUTLINE

Recombinant DNA is a form of DNA that does not exist naturally, in which a DNA fragment, such a gene, is introduced into an existing DNA, such as a bacteria plasmid [Jeremy M., *et al.*, 2007]. A recombinant protein is a protein that is derived from recombinant DNA.

The recombinant DNA technique was first proposed by Peter Lobban, and then realized by Cohen and others [Cohen SN., *et al.*, 1973]. This technique foreseed the gene isolation and amplification and gene insertion into bacteria. Recombinant DNA technology was made possible by the discovery, isolation and application of restriction endonucleases by Werner Arber, Daniel Nathans, and Hamilton Smith, for which they received the 1978 Nobel Prize in Medicine.

1.1.2 General strategy of gene cloning and recombinant protein production

The procedure to express the recombinant proteins foresee three steps:

1) *Identification of interest gene:* the gene coding for the protein object of study can come from genomic DNA or from a preexistent plasmid construct. Through a polymerase chain reaction (PCR) the gene is amplified and the restiction endonuclase sites are inserted into the sequence.

2) *Identification of the appropriate expression system*: the gene is inserted into the expression plasmid and the new construct is transfected into the host cells.

3) *Production of the recombinant*

4) *protein:* the protein expression can be constitutive or induced. The recombinant protein is then purified and used in appropriate tests.

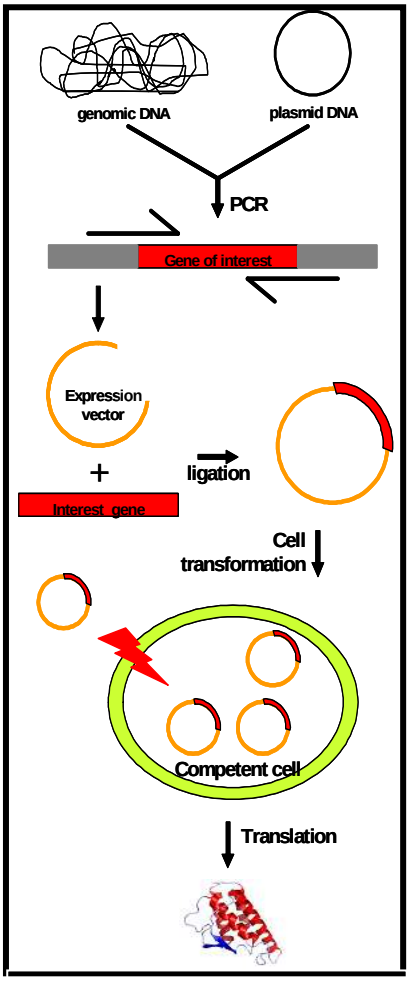


Fig. 1.1: Schematic diagram of the strategy applied for recombinant protein production.

1.2 IDENTIFICATION OF INTEREST GENE: THE IMMUNOTOXIN

Cancer remains the second most common cause of death in our society, and advanced disease is often refractory to surgical, chemotherapeutic, and radiologic interventions. Conventional therapies are often ineffective in eradicating the neoplastic disease, particularly in case of relapse. New biologic therapies may complement established therapeutic strategies.

1.2.1 DEFINITION OF IMMUNOTOXINS

The concept of Immunotoxins can be derived from the work of Paul Erlich in 1906. The Immunotoxins are protein toxins connected to a cell binding ligand of immunologic interest (Fig. 1.2). Classically Immunotoxins were created by chemically conjugating an antibody to a whole protein toxin, or, for more selective activity, by using a protein toxin devoid of its natural binding domain [Moolten FL, *et al.*, 1970; Krolick KA, *et al.*, 1980]. Proteins smaller than monoclonal antibodies (mAbs), like growth factors and cytokines, have also been chemically conjugated and genetically fused to protein toxins [Cawley DB, *et al.*, 1980].

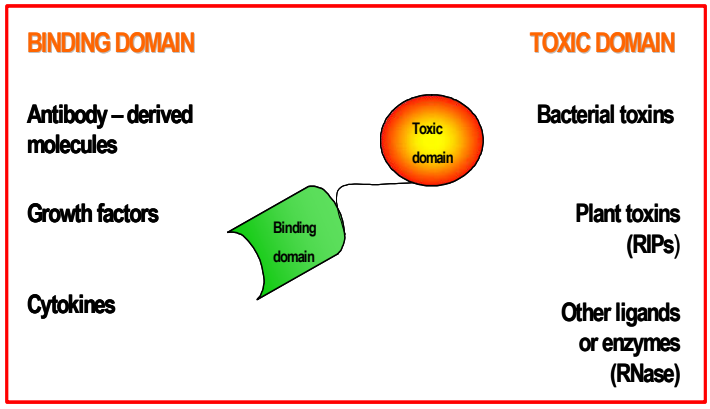


Fig. 1.2: Schematic diagram showing the Immunotoxin organization.

1.2.1.1 Immunotoxins compared with other surface-targeted therapies

One type of surface-targeted biologic therapy is represented by the use of "naked" monoclonal antibodies (mAb) (i.e. mAb used as such without any other therapeutic compound). Examples include rituximab [Akhtar S, *et al.*, 2002] and alemtuzumab [Keating MJ, *et al.*, 2002], which kill cells after binding. Humanized mAbs are clinically effective in up to half of the patients via

mechanisms of apoptosis induction, antibody-dependent cytotoxicity, and complement-dependent cytotoxicity.

Patients with malignant cells resistant to apoptosis, and patients whose immune system will not perform antibody- or complement- dependent cytotoxicity, may be resistant. To kill cells directly without relying on these mechanisms, a second type of surface-targeted therapy is used, one in which mAbs are conjugated to radionuclides. These agents induce responses in patients who are resistant to unlabeled mAbs [Cheson B, *et al.,* 2002]. However, radioimmunotherapy is limited by the potency of the radionuclide and the small number of radionuclide molecules that can be added to each mAb molecule.

A third type of surface-targeted therapy involves conjugating chemotherapy molecules to mAbs, which in many cases are more potent and cause less non-specific damage than radionuclides. Examples include gemtuzumab ozogamicin, a conjugate of an anti-CD33 mAb and calicheamicin [Nabhan C, *et al.,* 2002], which is approved for the treatment of acute myelogenous leukaemia (AML), and the anti-CD30-monomethyl auristatin E conjugate (cAC10-vcMMAE), under development for Hodgkin's disease (HD) and non-Hodgkin's lymphoma (NHL) [Francisco JA, *et al.,* 2003]. In the case of gemtuzumab ozogamicin, cells that are multi-drug resistant are, as would be expected, resistant to the target chemotherapeutic [Naito K, *et al.,* 2000]. Currently under development is a fourth type of surface-targeted therapy, which employs ribonucleases conjugated to mAbs [Hursey M, *et al.,* 2002].

Immunotoxins which are distinct from these approaches, target the surface of cancer cells with considerable potency, using protein toxins capable of killing a cell with a single molecule [Yamaizumi M, *et al.,* 1978; Eiklid K, *et al.,* 1980]. Irrespective of the cell cycle or cellular division, Immunotoxins can inactivate 200 ribosomes or elongation factor-2 (EF-2) molecules per minute. [Hall, W. A. and Fodstad, 1992; Hall, W. A. 1997] These potent proteins include plant toxins and single-chain bacterial toxins.[Carroll SF, *et al.,* 1987].

1.2.1.2 Production of Immunotoxins

Chemical conjugates of carrier portion and toxin generally involve either reducible disulfide (S-S) or non-reducible thioether (S-C) bonds [van Oosterhout

YV, *et al.,* 2001]. A thioether bond is appropriate if the ligand is conjugated to a toxin in the part that does not translocate to the cytosol, such as the binding domain [Pai LH, *et al.,* 1996]. Otherwise, a disulfide bond is commonly used.

Derivatization of the toxin requires only reduction in the case of ricin toxin A-chain (RTA) and its mutants, and also in the case of PE35, since both contain only one cysteine each. Derivatization of the ligand requires care to produce sulphydryls without harming the molecule, unless the ligand also has a single cysteine. Once the ligand and toxin are derivatized, they must be conjugated and the correct toxin-ligand ratio must be purified. The difficulty and cost of these multiple steps have pushed development of recombinant toxins, which may be produced in *Escherichia coli* transformed with a plasmid encoding the recombinant toxin. A common method of producing material for clinical trials is harvesting recombinant protein from insoluble bacterial inclusion bodies [Kreitman RJ, *et al.,* 1996; Kreitman RJ, *et al.,* 2000; Buchner J, *et al.,* 1992]. The insoluble protein can be washed extensively with detergent to remove endotoxin, solublized, denatured, and reduced. The recombinant protein is then renatured and purified by a chromatography column proper at the protein features. Other published methods of producing recombinant toxins from *E. coli* involve harvesting the protein from cytoplasm or cell lysate [Shao Y, *et al.,* 2001] and then using an affinity column to capture the diluted protein. Eukaryotic expression systems normally fail with recombinant toxins since eukaryotic EF-2 is highly susceptible to the toxin. However, insect, plant and yeast cells can produce active toxin [Choo AB, *et al.,* 2002; Woo JH, *et al.,* 2004] because during biosynthesis the toxin is driven in the secretory pathway and then accumulated in the culture medium.

1.2.1.3 Immunotoxins and clinical trials

Hematologic malignancies are optimal for treating with Immunotoxins, since malignant cells are often intravascular and accessible to intravenously administered drug, and since patients often lack sufficient immunity to make antibodies against the toxin. That said, only a handful of antigens have been used to target Immunotoxins to hematologic malignancies in patients. These are summarized in table 1.1.

Targeting solid tumors with Immunotoxins is much more difficult than targeting hematologic tumors. Not only are the cellular junctions tighter and the tumor cells more tightly packed, but the patients are less immunosuppressed and more likely to make neutralizing antibodies to the toxin. Below, recent published information regarding solid tumor Immunotoxin trials, along with recent preclinical development, is discussed. The findings are summarized in table 1.1.

Chemical Conjugates

Agent	Antigen	Ligand	Truncated Toxin	Basic Toxin	Diseases
RFT5-dgA	CD25	MAb	dgA	Ricin	HD
RFB4-dgA	CD22	MAb	dgA	Ricin	B-NHL, CLL
RFB4-Fab'-dgA	CD22	Fab'	dgA	Ricin	B-NHL
HD37-dgA	CD19	MAb	dgA	Ricin	B-NHL
Anti-CD7-dgA	CD7	MAb	dgA	Ricin	T-NHL
K_i-4.dgA	CD30	MAb	dgA	Ricin	HD
LMB-1	Le^y	MAb	Lys-PE38	PE	Carcinoma
TF-CRM107	TFR	Tf	CRM107	DT	Glioma
B43-PAP	CD19	MAb	PAP	PAP	ALL
Anti-B4-bRicin	CD19	MAb	bR	Ricin	B-NHL
Ber-H2-Sap6	CD30	MAb	Sap6	Saporin	HD
Anti-My9-bRicin	CD33	MAb	bR	Ricin	AML
454A12-rRA	TFR	MAb	rRA	Ricin	CSF cancer
N901-bR	CD56	MAb	bR	Ricin	SCLC

Recombinant toxins

Agent	Antigen	Ligand	Truncated Toxin	Basic Toxin	Diseases
Ontak	IL2R	IL-2	DAB_{389}	DT	CTCL, CLL, NHL
BL22	CD22	dsFv	PE38	PE	HCL, CLL, NHL
LMB-2	CD25	scFv	PE38	PE	NHL, leukemias
DT388-GM-CSF	GM-CSF	GM-CSF	DT388	DT	AML
B3(Fv)-PE38	Le^y	scFv	PE38	PE	Carcinoma
B3(dsFv)-PE38	Le^y	dsFv	PE38	PE	Carcinoma
TP40	EGFR	TGFα	$PE40^{4a}$	PE	Bladder cancer, CIS
TP38	EGFR	TGFα	PE38	PE	Glioblastoma
BR96(scFv)-PE40	Le^y	scFv	PE40	PE	Carcinoma
erb38	erbB2	dsFv	PE38	PE	Breast cancer
NBI-3001	IL4R	IL-4(38-37)	PE38KDEL	PE	Glioma
IL13-PE38QQR	IL13R	IL-13	PE38QQR	PE	Renal cell
SS1(dsFv)-PE38	Mesothelin	dsFv	PE38	PE	Mesothelioma
$DAB_{389}EGF$	EGFR	EGF	DAB_{389}	DT	Carcinoma

Table 1.1: Immunotoxins tested clinically in recent years.
(modified from Pastan, I *et al*., 2007).

Toxins include recombinant ricin A chain (rRA), blocked ricin (bR), deglycosylated ricin A chain (dgA), pokeweed antiviral protein (PAP), truncated diphtheria toxin (DT388 or DAB 389), truncated *Pseudomonas* exotoxin (PE38 or PE40), and mutated diphtheria toxin (CRM107). Non-monoclonal antibody (mAb) ligands include interleukin-2, -4, and -13 (IL-2, IL-4, and IL-13); granulocyte-macrophage colony stimulating factor (GM-CSF); epidermal growth factor (EGF); transforming growth factor (TGFα); and transferring (Tf). $PE40^{4A}$ is PE40 with alanine substituted for cysteine at positions 265, 287, 372, and 379. PE38QQR is PE38 with 2 glutamine residues and 1 arginine replacing the 3 lysine residues of PE38 at positions 590, 606, and 613. Diseases include non-Hodgkin's lymphoma (NHL, B- or T-cell), cutaneous T-cell lymphoma (CTCL), Hodgkin's disease (HD), chronic lymphocytic leukemia (CLL), carcinoma in situ (CIS), acute myelogenous leukemia (AML), metastatic tumor involving the cerebrospinal fluid (CSF cancer), renal cell carcinoma (renal cell), small cell lung cancer (SCLC), Acute lymphoblastic leukemia (ALL) and hairy cell leukemia (HCL).

1.2.2 TOXIC DOMAIN

The toxins that have been used to construct Immunotoxins are natural products of plants, bacteria, and fungi that all inactivate protein synthesis through different mechanisms (Table 1.2)

Toxins	Mechanism[a]
Plant	
Ricin	Inactivates ribosomes
Abrin	Inactivates ribosomes
Ricin A-chain	Inactivates ribosomes
Bacterial	
Diphtheria toxin	Catalyzes transfer of ADP-ribose to EF-2
Cross-reacting material 107	Catalyzes transfer of ADP-ribose to EF-2
Pseudomonas aeruginosa exotoxin A	Catalyzes transfer of ADP-ribose to EF-2
Pseudomonas aeruginosa exotoxin fragments (PE38, PE40)	Catalyzes transfer of ADP-ribose to EF-2
Fungal	
α-sarcin	Inactivates ribosomes

[a] ADP: adenosine diphosphate; EF: elongation factor.

Table 1.2: Immunotoxin mechanisms of action.
(Modified from Hall W. A., Methods in Molecular Biology, vol. 166: Immunotoxin Methods and Protocols. *Humana Press Inc*. Totowa, NJ)

1.2.2.1.Plant toxins

Plant holotoxins (also referred to as class II ribosome-inactivating proteins –RIPs II) include ricin, abrin, mistletoe lectin, and modeccin. Hemitoxins, or class I ribosome-inactivating proteins -RIPs I- , include PAP, saporin, bryodin 1, bouganin, and gelonin [Bolognesi A, *et al.*, 2000]. As shown in figure 1.3, holotoxins contain both binding and catalytic domains, whereas hemitoxins contain only catalytic domains.

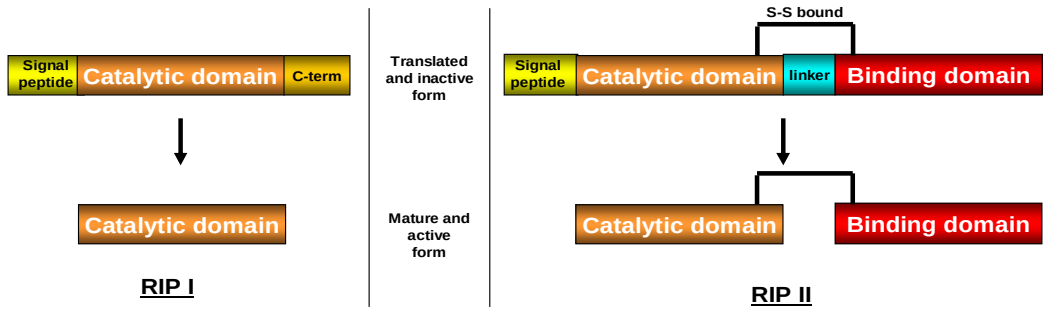


Fig. 1.3: **Schematic diagram showing the RIP I and RIP II organization.**
Holotoxins (RIP II) contain catalytic and binding domains disulfide-bonded together, while hemitoxins (RIP I) contain only catalityc domain.
The toxin inactive precursor is translated in the endoplasmic reticulum of plant cells. The loss of the signal peptide and the C-terminus makes the RIPs toxic.

1.2.2.1.1 Mechanism of action of plant toxins

The plant toxins have been shown to prevent the association of elongation factor-1 and -2 (EF-1 and EF-2) with the 60s ribosomal subunit by removing the A^{4324} in 28s rRNA [Endo Y, *et al.,* 1987]. Apoptosis has been shown to be involved in cell death induced by plant toxins [Bolognesi A, *et al.,* 1996; Hughes JN, *et al.,* 1996; Bergamaschi G, *et al.,* 1996]. Only the enzymatic domain of both holo- and hemitoxins translocates to the cytosol, so the binding domains of holotoxins must be removed by reduction of the disulfide bond prior to translocation. Exactly how plant toxins move from the cell surface to the cytosol is unknown; the process probably differs for each plant toxin.

The intracellular transport of ricin is dependent on sorting receptors that cycle between the endoplasmic reticulum (ER) and the terminal compartments of the Golgi [Wesche J, *et al.,* 1999]. It has been shown that glycolipids that bind ricin may be transported from endosomes to the Golgi and that the Lysine-aspartic acid-glutamic acid-Leucine (KDEL) ER retention sequence, if added to ricin, enhances the delivery of this plant toxin to the cytosol [Tagge E, *et al.,* 1997].

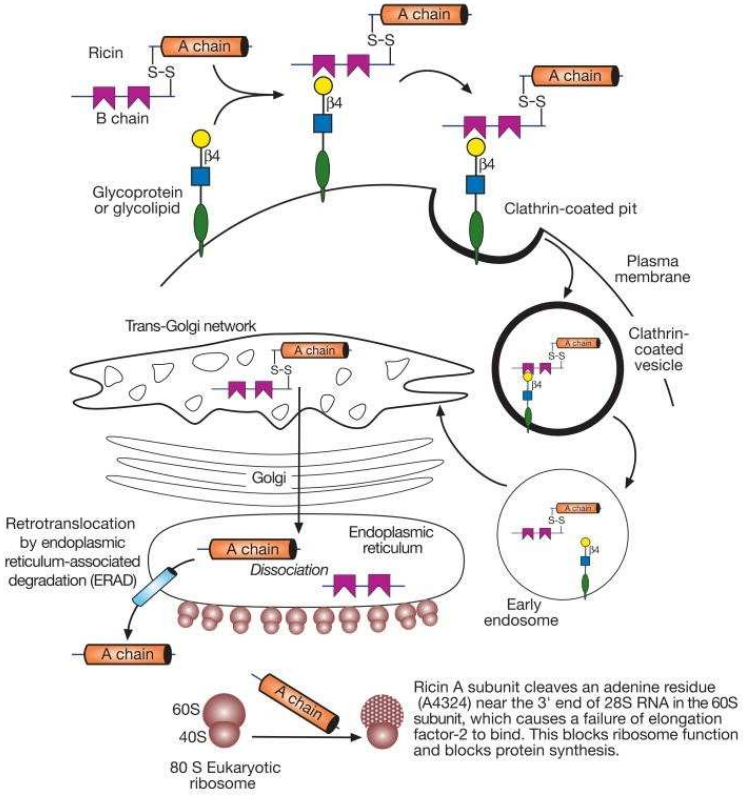


Fig. 1.4: Mechanism of ricin internalization and intoxication.
From www.ncbi.nlm.nih.gov/bookshelf/

1.2.2.1.2 Attempts to construct fusion toxins using plant toxins

The cytotoxicity of both plant and bacterial toxins is optimal when the catalytic domain alone translocates to the cytosol [Mohanraj D, *et al.,* 1995] . A binding domain can be translocated to the cytosol if placed within the catalytic domain, but cytotoxic activity is significantly reduced [Kreitman RJ., *et al.,* 1992].

In the '90s several recombinat toxins were constructed using portions of plant toxins such as the A-chain of ricin (RTA) [Cook JP, *et al.,* 1993] or whole toxins such as PAP [Dore JM, *et al.,* 1997] or SAP [Fabbrini MS, *et al.,* 1997; Tetzke TA, *et al.,* 1997]. For these molecules, it is not known whether (1) the recombinant toxin enters the cytosol of target cells intact, or (2) the ligand is unstable after internalization, permitting the catalytic domain alone to translocate to the cytosol. The ability of even stable ligands to predictably separate from the catalytic domain is an important feature of recombinant toxins [Kreitman RJ, 1997] and a unique feature among all toxins provided by the bacterial toxins PE and DT.

1.2.2.2 Bacterial Toxins

1.2.2.2.1 Mechanism of Action of Bacterial Toxins

Both the *Pseudomonas* exotoxin A (PE) and the diphtheria toxin (DT) enzymatically ADP-ribosylate EF-2 in the cytosol [Carroll SF, *et al.,* 1987]. They each catalyze the ADP-ribosylation of histidine-699 of EF-2, which is post-translationally modified to a diphthimide residue [Phan LD, *et al.,* 1993]. Despite their similar action, PE and DT differ greatly in their amino acid sequence, and in fact PE's enzymatic domain is near the carboxyl terminus, while DT's is near the amino terminus. Conversely, PE's binding domain is near its amino terminus, and DT's is near its carboxyl terminus (Fig.1.5).

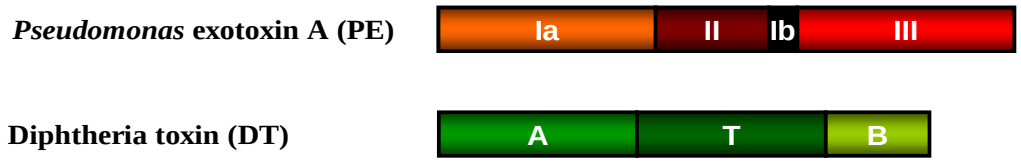


Fig. 1.5: Schematic structure of Pseudomonas exotoxin A (PE) and Diphtheria toxin (DT).

1.2.2.2.2 Mechanism of intoxication of PE

Full-length 613-amino-acid PE, as shown in figure 1.5, is a single-chain protein containing 3 functional domains [Hwang J, *et al.,* 1987; Allured VS, *et al.,* 1986]. Domain Ia (amino acids 1-252) is the binding domain, domain II (amino acids 253-364) is responsible for translocating the toxin to the cytosol, and domain III (amino acids 400-613) contains the ADP-ribosylating enzyme that inactivates EF-2 in the cytosol. The catalytic process of ADP-ribosylation has been shown to involve the His440 and Glu553 toxin residues [Li M, *et al.,* 1996]. His440 binds nicotinamide adenine dinucleotide (NAD) via Adenosine monophosphate (AMP) ribose. The carboxyl group of the Glu553 side chain, through a water-mediated hydrogen bond with Tyr481 and Glu546, allows Tyr481 to bind NAD through a ring-stacking mechanism. The function of domain Ib (amino acids 365-399) is unknown. Thus, a current model of how PE kills cells contains the following steps (Fig. 1.6) :

(1) The C-terminal residue (Lys613) is removed by a carboxypeptidase in the plasma or culture medium [Hessler JL, *et al.,* 1997].

(2) Domain Ia binds to the a 2-macroglobulin receptor that is present on animal cells and is internalized via endosomes to the trans-reticular Golgi [Kounnas MZ., *et al.,* 1992].

(3) After internalization, the protease furin cleaves domain II between amino acids 279 and 280 [Chiron MF, *et al.,* 1994].

(4) The disulfide bond between cysteines 265 and 287, which joins the 2 fragments generated by proteolysis, is reduced [McKee ML, *et al.,* 1999].

(5) Amino acids 609 to 612 Arginine - glutamic acid - aspartic acid - leucine (REDL) bind to an intracellular sorting receptor that transports the 37 kDa carboxy terminal fragment from the trans-reticular Golgi apparatus to the ER [Chaudhary VK, *et al.,* 1995; Kreitman RJ, *et al.,* 1995].

(6) Amino acids 280 to 313 mediate translocation of the toxin to the cytosol [Theuer C, 1994; Theuer CP, *et al.,* 1993].

(7) The ADP-ribosylating enzyme within amino acids 400 to 602 inactivates EF-2 [Carroll SF, *et al.,* 1987].

(8) While inhibition of protein synthesis is sufficient to induce cell death eventually, recent experiments indicate that cell death from toxins is facilitated by apoptosis [Keppler-Hafkemeyer A, *et al.,* 2000; Brinkmann U, *et al.*, 1995].

1.2.2.2.3 Mechanism of intoxication of DT

DT is a single-chain protein 535 amino acids in length. It is composed of an enzymatic A domain (amino acids 1-193) and a binding B domain (amino acids 482-535) [Rolf JM, *et al.,* 1990]. A third domain, which is the translocation or transmembrane (T) domain, is located in the center of the molecule [Choe S, *et al.,* 1992] (Fig. 1.5). Based on DT's 3-dimensional structure in the presence and absence of NAD [Bell CE , *et al.,* 1996], DT is thought to undergo these steps to kill cells (Fig. 1.6):

(1) DT is proteolytically cleaved outside the cell between Arg193 and Ser194 [Williams DP, *et al.,* 1990], which is within a disulfide loop formed by Cys186 and Cys201.

(2) DT binds on the cell surface via residues 482 to 535 to a complex of heparin-binding Epidermal growth factor (EGF)-like growth factor precursor and CD9 [Rolf JM, *et al.,* 1990].

(3) DT internalizes into an endosome and unfolds at low pH [D'Silva PR, *et al.*, 1998], and the disulfide bond linking amino acids 186 and 201 is reduced.

(4) The TH8 (amino acids 326-347) and TH9 (amino acids 358-376) domains form a hairpin, which inserts into the membrane of the endosome and forms a channel through which the enzymatic fragment translocates to the cytosol [Kaul P, *et al.,* 1996], probably from early endosomes [Lemichez E, *et al.,* 1997].

(5) In the cytosol, NAD binds to the active-site cleft of DT (amino acids 34-52), and the ADP ribose of NAD is transferred to EF-2 [Wilson BA, *et al.,* 1994; Bennett MJ, *et al.,* 1987].

(6) As with PE, cell death is facilitated by apoptosis [Brinkmann U, *et al.,* 1995].

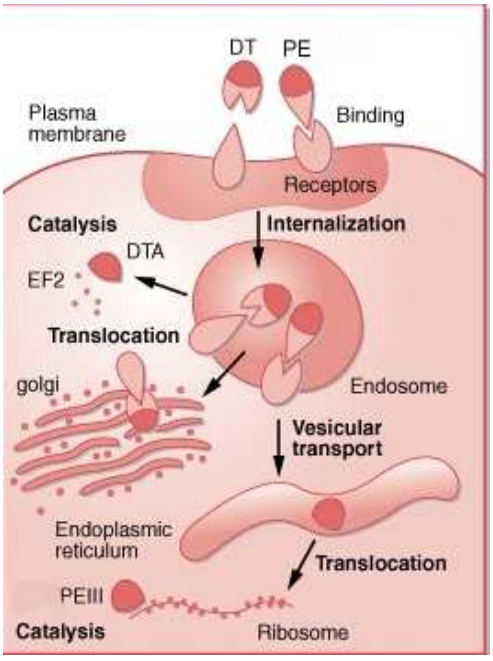


Fig. 1.6: Mechanism of intoxication of DT and PE

1.2.2.2.4 Mutated bacterial toxins for fusing to ligands

The structures of mutated and truncated forms of DT and PE are shown in figure 1.7. To improve specificity, toxins for labeling mAbs are mutated to prevent their binding to normal cells. DT is mutated by converting Leu390 and Ser525 each to phenylalanine, resulting in CRM107 [Greenfield L, *et al.,* 1987]. Truncated forms of PE and DT include PE40, containing amino acids 253 to 613 of PE, and Diptheria toxin A and B domains (DAB 486), containing the first 485 amino acids of DT [Hwang J, *et al.,* 1987; Kondo T, *et al.,* 1988; Williams DP, *et al.,* 1987]. Shorter versions more recently used include PE38, composed of amino acids 253 to 364 and 381 to 613 of PE, and DT388 or DAB 389, containing the first 388 amino acids of DT [Siegall CB, *et al.,* 1989; Kreitman RJ, *et al.,* 1993; Williams DP, *et al.,* 1990; Chaudhary VK, *et al.,* 1991]. To allow the ADP-ribosylating domain to translocate to the cytosol without the ligand, the ligand is placed at the amino terminus of PE and at the carboxyl terminus of DT. Another form of PE has an altered carboxyl terminus from the Arginine-glutamic acid-aspartic acid-leucine-lysine (REDLK) to the KDEL sequence, which binds with higher affinity to the KDEL receptor and results in increased cytotoxicity [Kreitman RJ, *et al.,* 1995]. Immunotoxins containing mutants of PE ending in KDEL (ie, PE38KDEL or PE40KDEL) are more cytotoxic than comparable

Immunotoxins where the PE mutant ends in the native sequence REDLK [Kreitman RJ, *et al.*, 1995; Kreitman RJ, *et al.*, 1993; Seetharam S, *et al.*, 1991]. The translocated fragment of PE38 is 35 kDa in length beginning with Gly280, and since methionine in this position does not alter activity, the new mutant PE35 is produced; it begins with a methionine at position 280 and contains amino acids 281 to 364 and 381 to 613 [Theuer CP, 1993]. This molecule would not be appropriate for fusing to ligands, but since it contains a single disulfide bond, it is ideal for chemically conjugating to ligands.

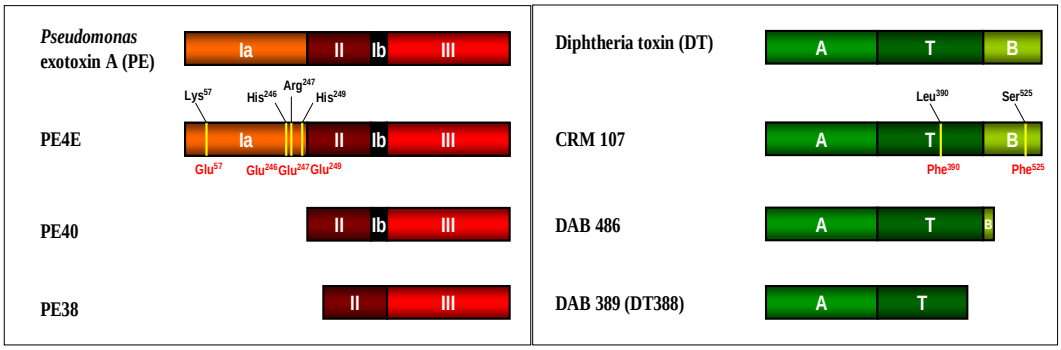


Fig. 1.7: Schematic structure of mutated bacterial toxins for fusing to ligands

1.2.3 THE BINDING DOMAIN

In the design of an Immunotoxin a variety of binding domains can be used to selectively deliver the drug to the intended cell target; besides monoclonal antibodies and fragments thence derived, other small proteins are appropriate to fulfil this function, e.g. growth factors and cytokines. Such molecules impart specificity to the Immunotoxin by virtue of the overexpression of antigen tumor associated on the plasma membrane and some receptors for growth factors, cytokines on tumor cells [Kreitman, R. J. 2006].

1.2.3.1 Antibodies and its fragments

Antibodies are oligomeric proteins belonging to the immunoglobulin (Ig) superfamily and represent the characterizing element of the humoral adaptive immune system.

The basic structure of an antibody comprises four polipeptidic chains: two identical light chains of approximately 25 kDa in size and two identical heavy chains of 50 kDa. Disulphides and weak molecular interactions link two heavy chain – light chain heterodimers yielding the basic structure of a four-chain

antibody of 150 kDa. The variable domains of the light (V_L) and heavy (V_H) chains together form the antigen binding site; the constant regions of the two heavy chains constitute the so-called Fragment Crystallizable (Fc) portion which is responsible for the effector functions of the antibody, and it also contributes to the *in vivo* structural stability of the antiboig (Fig. 1.8) ["Kuby Immunology 5th edition", Goldsby, R. A. *et al*., ed. Goldsby, R. A., 2003].

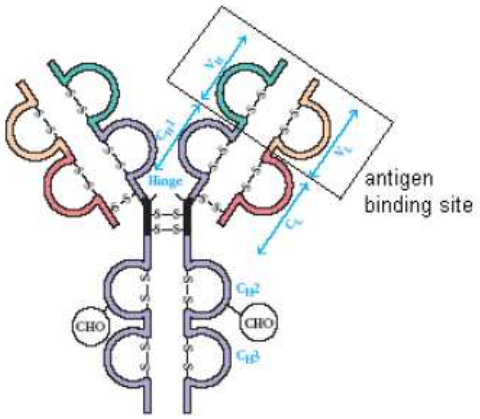


Fig. 1.8: Diagram of the antibody structure.
[Modified from Goldsby, R. A. *et al*., 2003].

Initially antibodies were purified from the serum of animals priorly immunized with an antigen yielding a polyclonal pool of reactive immunoglobulins. Despite their high binding affinity, polyclonal antibodies are unsuitable for therapeutic use, due to their heterogeneous composition. The production of monoclonal antibodies (mAbs) was made possible by the introduction of the hybridoma technology, a historical breakthrough for immunology and the emerging field of immunotherapeutics. The direct clinical employment of murine mAbs is hindered by the short half-life in the bloodstream and by the strong immunogenicity triggered in humans. The inevitable immune response of the patient to the mouse protein (also referred to as HAMA, "Human Anti-Mouse Antibody") leads to a rapid clearance of the mAb and is often accompanied by hypersensitivity reactions [Schrama, D. *et al.*, 2006].

The drawbacks of murine mAbs have been overcome with the introduction of chimeric or humanized mAbs (Fig. 1.9). Chimerization involves the linking of the variable domains from a murine mAb to the constant portions of a human immunoglobulin. The advances of molecular biology have allowed the construction of hybrid genes that code for antibody molecules in which variable (V) and constant (C) regions are of murine and human origin, respectively [Carter, P. J., 2006]. To obtain humanized antibodies the sequences coding for the sole murine CDRs are "grafted" onto a human IgG [Carter, P. J., 2006].

A growing number of antibodies now in clinical trials are fully human, as they are generated either from IgG-*knockout* transgenic mice bearing the genes for human immunoglobulins or from the employment of new technologies like *phage-display* [Lonberg, N., 2005; Konthur, Z., 2005].

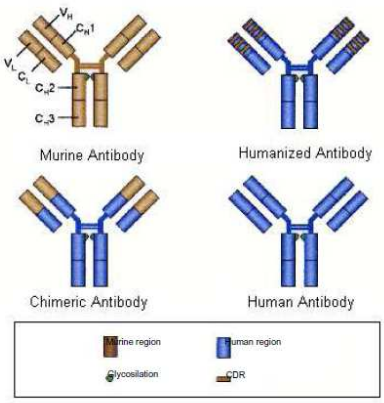


Fig. 1.9: Schematic diagram of fully murine, chimeric, humanized and fully human antibodies.

Alternative strategies for generating human mAbs are for instance the obtainment of hybridomas from patient's lymphocytes, the cloning of an immunoglobulin cDNA from a single lymphocyte or other methods like *ribosome-*, mRNA-, or *yeast- display* [Carter, P. J., 2006].

The employment of whole immunoglobulins as carrier molecules to deliver a toxin to cancerous cells may imply a certain degree of non selective activity for an IT, owing to non-specific binding of the antibody Fc portion. Furthermore, the big size of these molecules can hinder their penetration in tumor tissue, especially in the case of solid tumors. As a consequence antibody fragments were developed. They are smaller molecules that still maintain an unaltered binding capability and specificity to the antigen [Plückthun, A., 1992]. In figure 1.10 several examples of different types of antibody fragments were reported [Plückthun, A., 1992; Holliger, P. and Hudson, P. J., 2005].

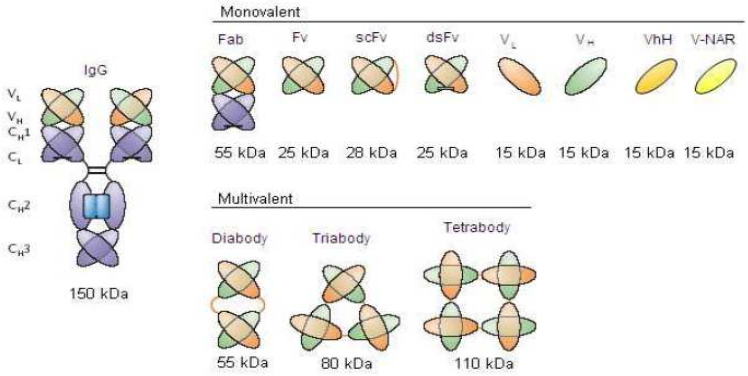


Fig. 1.10: Schematic representation of different possible antibody configurations. The domain-based structure of immunoglobulins could be manipulated to yield a wide repertoire of antibody formats ranging from 15 kDa to 100 kDa insize. [Modified from Carter, P. J., 2006].

1.2.3.2 Cytokines and growth factors

Other ligands utilized for building Immunotoxins are the cytokines and the growth factors; although these bind also to normal cells, yours receptors are overexpressed in several hematological and solid tumour cells. Cytokines and growth factors are effective targeting agents because their affinity for their cell surface receptors can be higher than that of monoclonal antibodies. Moreover the receptors of these molecules are able to internalise yours ligand, and than also the respective cytokine/growth factors-based Immunotoxin with great efficiency by receptor-mediated endocytosis [Kelley, V. E., et al., 1988]. Furthermore, being of human origin they are non immunogenic.

Possible drawbacks of using cytokines and growth factors as carrier molecules is their rapid clearance *in vivo* and the agonistic effects often exerted by these molecules linked to the toxic moiety, witch could promote proliferation of the targeted cells when the amount of Immunotoxins bound are insufficient to kill the target cell [Fracasso, G., et al., 2004]. A further disadvantage is the presence of circulating ligands or soluble receptors that compete for the IT [Fracasso, G., et al., 2004]. The cytokines/growth factors that were used as targeting agents are IL-2, IL-4 and IL-6 [Chadwick, D. E., *et al.*, 1993; Ogata, M., *et al.*, 1989;Walz, G., *et al*, 1989], TNF [Gould, D. J., *et al*., 1998; Hoogenboom, H. R., *et al.*, 1991], epidermal growth factors (EGF) [Chandler, L. A., *et al.*, 1998;Di Massimo, A. M., *et al*., 1997; Hirota, N., *et al*., 1989; Matsui, H., *et al.*, 1989; Ozawa, S., *et al*., 1989; Banker, D. E., *et al*., 1989; Volmar, A. M., *et al*., 1987], fibroblast growth factor (FGF) [Davol, P., *et al.*, 1995; Dazert, S., et al., 1998; Lappi, D.A., *et al*., 1995; Lin, P.H., *et al*., 1998], granulocyte-macrophage colonise stimulating factor (GM-CSF) [Lappi D. A., *et al*., 1993; Burbage, C., *et al*., 1997], transferrin (Tfn) [Shannon, K. M., *et al*., 1990; Candiani C., *et al*., 1992; Chignola, R., Anselmi, C., *et al*., 1995; Chignola, R., Foroni, R., *et al.*, 1995; Colombatti, M., *et al.*, 1990; Nicholls, P. J., *et al.*, 1993] and nerve growth factor (NGF) [Benedetti, G., *et al*., 1994; Book, A. A., *et al*., 1992; Rossner, S., *et al*., 1994; Wiley, R. G., *et al*., 1995].

1.2.3.2.1 IL4

Interleukin-4 (IL-4) is a pleiotropic Th2-derived immune cytokine, which is predominantly produced by activated T lymphocytes, mast cells, and basophils [Paul WE, 1991; Puri RK, *et al.*, 1993; Puri RK, 1995]. IL-4 has been shown to have various activities in many different cell types, such as T cells, B cells, monocytes, endothelial cells, and fibroblasts [Paul WE, 1991; Puri RK, *et al.*, 1993; Puri RK, 1995]. To induce biological activities, IL-4 must bind to its specific receptor, which is generally present on the plasma membrane of target cells. IL-4 by itself has been shown to have modest antiproliferative activity on hematopoietic malignant cell lines and solid tumor cell lines *in vitro* and *in vivo* [Toi M , *et al*., 1992; Topp MS, *et al*., 1993].

1.2.3.2.1.1 IL4-Receptor (IL4R)

IL-4 receptors are overexpressed on a variety of hematologic and solid malignant tumor cell lines including malignant melanoma, breast carcinoma, pancreatic tumour, ovarian carcinoma, esothelioma, glioblastoma, renal cell carcinoma, head and neck carcinoma, and AIDS-associated Kaposi's sarcoma [Puri, R. K.,et al., 1993; Puri, R. K.,et al., 1995; Puri, R. K., et al., 1991; Obiri, N., et al., 1993; Obiri, N., et al., 1994; Hussain, S. R., et al., 1994; Hoon, S. R., at al., 1997; Toi, M., et al., 1992; Morisaki, T., et al., 1992; Puri, R. K.,1996; Kreitman, R. J., et al., 1994; Kawakami, K., et al., 2000; Puri, R. K., et al. 1996; Leland, P., et al., 2000; Gallizi, J. P., et al., 1989]. Normal cells including hematopoietic cells (e.g. resting T cells, B cells, and monocytes), fibroblasts, and endothelial cells also expressed IL-4R [Paul WE, 1991; Puri RK, *et al.*, 1993; Puri RK, 1995; Puri RK, *et al*., 1994; Puri RK, et al., 1996]; however, receptor numbers were significantly lower compared to most tumor cell lines.

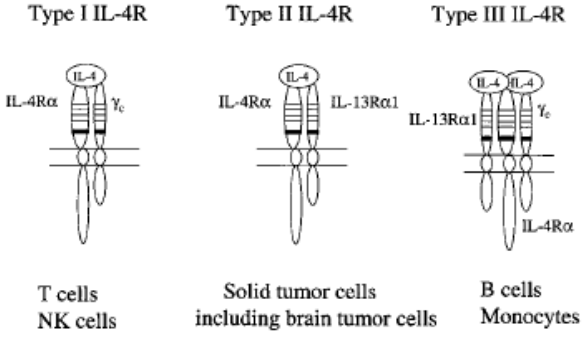


Fig. 1.11: Schematic model of IL4R subunits.
Modified from Kawakami M, *et al*., 2003.

Several studies suggested that the immune cells express type I IL-4R

(IL-4Rα and IL-2Rγ chains) [Russell SM, *et al*., 1993; Kondo M, *et al.*, 1993; Murata T, *et al.*, 1995], and tumor cells express type II IL-4R (IL-4Rα and IL-13Rα1 chains) [Kawakami K, *et al.*, 2000; Murata T, *et al.*, 1995; Obiri NI, *et al.*, 1995; Obiri NI, *et al.*, 1994]. Some cells instead express all three (IL-4Rα, IL-2Rγ, and IL-13Rα1) chains (type III IL-4R), however, whether all three chains form an IL-4R complex in immune or tumor cells is not clearly known as yet (Fig. 1.11).

Although the significance of overexpression of IL-4R on solid tumor cell lines is still not known, the advantage of overexpression of these receptors has taken by designing a receptor targeted cytotoxic agent for possible cancer therapy. A recombinant fusion proteins comprised of IL-4 and *Pseudomonas* exotoxin has produced for possible treatment of recurrent human glioma. [Puri RK, *et al.*, 1994].

1.2.4 RECOMBINANT FUSION PROTEIN BETWEEN INTERLEUKIN-4 AND *Pseudomonas* EXOTOXIN (IL4-PE)

In 1989, Ogata Masato and colleagues first cloned a recombinant Immunotoxin in witch the cell binding domain of PE was replaced by murine IL-4 (IL4-PE40) [Ogata M., et al., 1989]. The fusion gene product, was found to be highly toxic to a murine T-cell line (CT.4R) but to have no effect on human cell lines lacking receptors for murine IL-4. In addition, a chimeric protein composed of a mutant form of PE40 that had very low ADP-ribosylating activity (PE40 asp553) had displayed mitogenic activity similar to that of IL-4 rather than cytotoxic activity, showing that ADP-ribosylating activity was essential for cytotoxicity. Moreover an excess amount of IL4 or a neutralizing monoclonal antibody to IL-4 blocked the cytotoxicity of IL4-PE40 [Ogata M., *et al*., 1989].

In 1991, Puri Raj K. and co-workers demonstrated the presence of IL4 receptor on murine solid tumors of nonlymphoid origin and the IL4-PE40 ability to inhibit the protein synthesis in these cells. The specificity of action was demonstrated through the IL4 receptor blocking [Puri, R.K., *et al.*, 1991].

Because murine IL4 does not bind to the human IL-4R [Morrison, B., *et al*., 1992], it was not possible to evaluate the activity of these chimeric toxins on human cells. In 1993, Debrinski Waldemar and co-workers constructed the

chimeric toxin with the human IL-4 fuses to a gene coding a mutant form of PE in which 4 amino acid residues (Lys^{57}, His^{246}, Arg^{247} and His^{249}), all in domain Ia of PE, are changed to glutamates (PE4E). This mutation abolishes the binding of PE to its receptor [Chaudhary VK, et al., 1990]. Use of different type of IL4R+ target cells (i.e. carcinomas of the lung, kidney, ovary, breast, colon [Obiri, N. I., et al, 1993; Puri RK, et al., 1993; Tungekar, M. F., et al., 1991], malignant stomach, liver, prostate, adrenal, and cervix as well as melanoma and epidermoid carcinoma [Debinski W, *et al*., 1993]) has demonstrated that IL4-PE4E cytotoxicity is mediated by the presence of the IL4R. Other works documented the ability of hIL4 to eradicate tumors in immunocompetent animals [Golumhek, P. T., *et al.,* 1992; Tepper, R. I., *et al.,* 1989].

IL4 has been originally identified as a B cell growth factor and it has been shown to affect a variety of hematopoietic cells, such as T lymphocytes [Yokota, T., *et al.,* 1986]. The activation of peripheral blood lymphocytes increases the number of hIL4R on blood cells. hIL4-PE4E was very cytotoxic to PHA-activated peripheral blood lymphocytes, indicating the possibility of the use of this chimeric toxin for intervention in some specific immune responses. Furthermore, hIL4-PE4E may diminish antibody responses against itself by targeting cells participating in the immune response [Debinski W, *et al*., 1993].

The Immunotoxin design proceeded with the carrier affinity and specificity optimization and the development of cytotoxic activity of toxin. Often fusion of one protein to another impairs the activity of one or both proteins. This can occur if one or both proteins require one or both termini free for optimal activity. Alternatively, connection of one protein to another can impair the ability of either protein to properly fold. It has been reported that transforming growth factor α, IL2, IL3, IL4, IL6, GM-CSF, and insulin-like growth factor I all bind with 20- to 250-fold reduced affinity after fusion to another protein [Curtis, B. M, *et al*., 1991; Edwards, G. M., *et al*., 1989; Debinski W., *et al*., 1993; Williams DP , *et al*., 1990; Prior, T. I., *et al*., 1991]. At the beginning, the options available for improving the function of a fusion protein were limited to switching the order of the two proteins, attaching linkers in between the two proteins, or mutating one of the proteins to decrease junctional effects [Edwards, G. M., *et al*., 1989; Brinkmann, U, *et al*., 1992]. When the hIL4 is fused with the fragment or whole

PE, the chimeric proteins bind to the IL4R with only ~ l% of the affinity of native IL4 [Debinski W., *et al*., 1993;]. Several studies have indicated that the carboxyl terminus of IL4 is important for binding [Le, H. V , 1991; Ramanathan, L , 1993]; therefore, it is likely that the large toxin molecule attached to the carboxyl terminus blocks the binding of IL4 to the IL4R. A new strategy for fusing the two proteins is developed, in which the toxin is fused to the new carboxyl terminus of circularly permuted forms of IL4. A circular permuted protein is a mutant protein in which the termini have been fused and new termini created elsewhere in the molecule. Several circularly permuted proteins have been made but none of these have been fused to other proteins [Pan, T. & Uhlenbeck, O. C.,1993].

The strategy for construction of the DNA encoding CP-IL4 was to amplify the first and second parts of IL4 sequence, so that the 5' end of the first fragment and the 3' end of the second fragment are identical and encode the GGNGG linker. The two fragments therefore anneal in reverse order to form a template for the final PCR step with the final PCR product CP-IL4. With this strategy two differets CP-IL4 were constructed: IL4(38-37) and IL4(105-104). [Kreitman RJ, *et al.*, 1994] (Fig. 1.12).

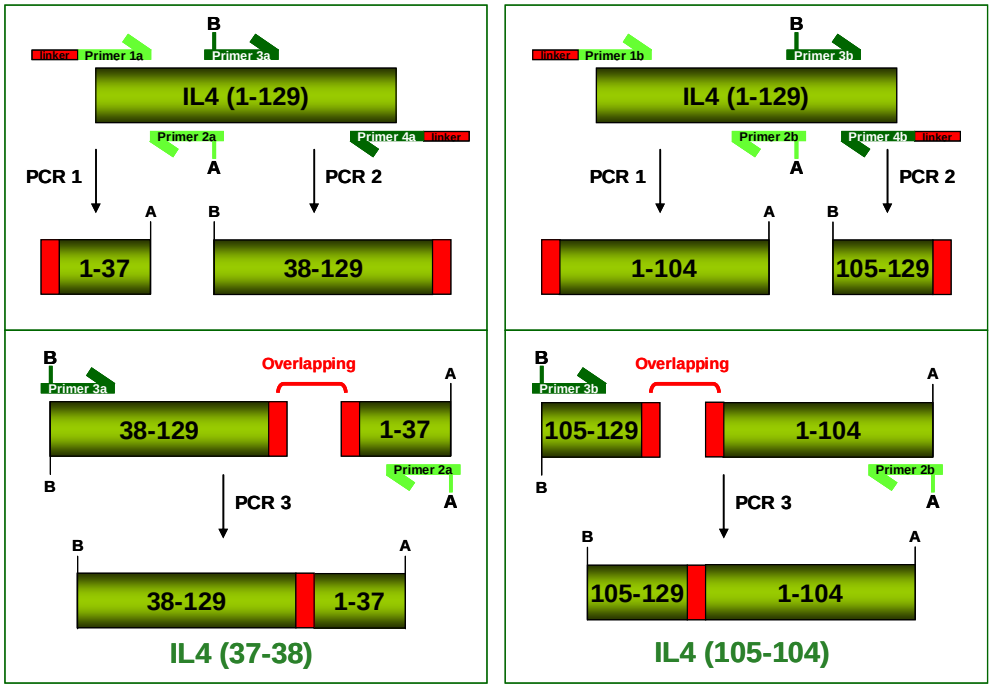


Fig. 1.12: Construction of circularly permuted forms of IL4 (CP-IL4)

The circularly permuted IL4 (CP-IL4) was fused to a PE so that the junction would be in an entirely new location and allow the IL4 to bind in a more

native fashion [Kreitman RJ, *et al.*, 1994]. The CP-IL4-PE38 study showed that IL4(37-38)-toxin bound the IL4R positive cells better than IL4(105-104)-toxin. Moreover the toxin fused to the new carboxyl terminus (amino acid 37) of CP-IL4 improved binding about 10-fold over a molecule in which the toxin was fused to the native carboxyl terminus (amino acid 129) of IL4. The new junction site decreased the impairment in IL4R-binding. [Kreitman RJ, *et al.*, 1994].

1.2.4.1 Clinical trials of IL4-PE recombinant Immunotoxin

The first attempts to use the CP-IL4-PE38 in clinical trials are described in 1993 by Theuer C.P. and Pastan I. [Theuer C.P., *et al*., 1993]. With the first chemical Immunotoxin produced clinical results were disappointing. But the recombinant Immunotoxins circumvented problems such as immunogenicity that may limit the clinical usefulness.

Afterwards, in 2000, Rand R.N. described the intratumoral administration of CP-IL4-PE38 Immunotoxin in patient with high-grade glioma based on the consideration that human glioblastoma cells but not normal brain cells express numerous receptors for IL4 [Rand R.N., *et al*., 2000].

Several studies were conducted in the phase I/II clinical trials to CP-IL4-PE38 as treatment against glioblastoma tumor, testing the maximal tolerated concentration, the better volume administrated [Rand R.N., *et al*., 2000], the administration way (intratumoral or intravenously) [Garland L., *et al*., 2005], the infusate viscosity to increase the volume of distribution [Mardor Y, *et al.*, 2009]. These studies showed the efficacy of Immunotoxin to reducing or remitting the glioma solid cancer [Kawakami, K, *et al*., 2001; Kawakami M., *et al*., 2002; Weber F.W, *et al*., 2003; Kawakami M., *et al*., 2003; Garland L., *et al*., 2005; Shimamura T., *et al*., 2006, Rainov N.G., *et al*., 2006; Mardor Y, *et al.*, 2009; Puri S., *et al.*, 2009].

In 2009, Oh S. described the possibility to use a bispecific targeted toxin simultaneously recognizing human epidermal growth factor and interleukin-4 receptors to treat the metastatic breast carcinoma in a mouse model. [Oh S., *et al*., 2009]. This bispecific molecule was also mutagenized to reduced the toxin immunogenicity. The bispecific ligand-directed toxin (BLT) was created cloning both human epidermal growth factor (EGF) and interleukin-4 cytokines onto the

same single-chain molecule with truncated Pseudomonas exotoxin (PE(38)). Site-specific mutagenesis was used to mutate amino acids in seven key epitopic toxin regions that dictate B-cell generation of neutralizing antitoxin antibodies. The BLT Immunotoxin was significantly effective against established systemic human breast cancer and prevented metastatic spread. Moreover the mutagenesis reduced immunogenicity by approximately 90% with no apparent loss in in vitro or in vivo activity [Oh S., *et al*., 2009].

1.2.5 PROBLEMS AND OPPORTUNITIES IN IMMUNOTOXIN DEVELOPMENT

The problems, including immunogenicity, unwanted toxicity, difficulty in production, limited half-life, and resistance, will be considered below, along with potential opportunities for improved development of Immunotoxins.

1.2.5.1 Immunogenicity

Based on a wide range of clinical trials, the incidence of immunogenicity after a single cycle of Immunotoxin ranges from 50% to 100% for solid tumors, and from 0% to 40% for hematologic tumors [Olsen E, *et al.,* 2001]. The immune response against the Immunotoxins leads to the appearance of measurable of neutralizing anti-IT antibodies. These antibodies lower the levels of biologically active Immunotoxin and compromises efficacy. Several approaches can be used to prevent immunogenicity. The method most useful is PEGylation, which not only blocks immunogenicity but also prolongs half-life [Onda M, *et al.,* 2003; Tsutsumi Y, *et al.,* 2000]. Immunologic studies have found a large number of B-cell and T-cell epitopes on *Pseudomonas* exotoxin [Roscoe DM, *et al.,* 1997; Roscoe DM, *et al.,* 1994; Nagata S, *et al.,* 2004]. The mutagenesis of 7 amino acid contained in 7 key epitopic toxin regions that dictate B-cell generation of neutralizing antitoxin antibodies reduced immunogenicity by approximately 90% with no apparent loss in *in vitro* or *in vivo* activity [Oh S., *et al*., 2009].

1.2.5.2 Unwanted Toxicity

A variety of toxicities have been observed with Immunotoxins that have limited the dose and hence the efficacy. The most common toxicity is the vascular

leak syndrome (VLS) that is characterized by an increase in vascular permeability resulting in tissue edema and, ultimately, multiple organ failure [Baluna R. and Vitetta E.S., 1997].

Studies have shown that RTA binds directly to endothelial cells, while truncated PE requires a ligand that cross-reacts with the endothelium [Kuan C, *et al*., 1995]. Such studies led to a mutant form of RTA that shows less VLS in an animal model [Kreitman RJ, *et al*., 1993].

Hepatotoxicity, a typical side effect of recombinant Immunotoxins, is attributed to the binding of basic residues on the Fv to negatively charged hepatic cells [Schnell R, *et al*., 1998, Onda M, *et al*., 2001]. Renal toxicity due to Immunotoxins is less well defined and could be non-specific at least in part because the kidneys are the dominant route of excretion of recombinant Immunotoxin [Huston JS, *et al*., 1988].

1.2.5.3 Difficulty in Production

Originally, chemical conjugates were made for clinical trials since manufacturers of recombinant toxins faced problems of endotoxin contamination and low yield. Advances in the production of other recombinant proteins for clinical use have solved many of these problems and have allowed large-scale production of recombinant toxins in prokaryotic system with high purity and reasonable cost. The optimization of an eukaryotic system to Immunotoxin expression could exceed the endotoxin contamination problem with reduction of production time maintaining low the production costs.

1.2.5.4 Potential for Future Development

For many types of disease, Immunotoxins are unlikely to work by themselves. Their half-lives may be too limited for diffusion to occur into solid tumor masses. It is possible that combination with other therapeutic agents having non-overlapping toxicities will result in better responses. Similarly, treatment of microscopic disease may be useful after cytoreduction by surgery, chemotherapy, or radiotherapy.

1.3 IDENTIFICATION OF THE APPROPRIATE EXPRESSION SYSTEM

1.3.1 PROKARIOTIC EXPRESSION SYSTEM

The recombinant expression of genes is of great interest for both biotechnology and basic research in the context of protein function and structure. The prokaryotic expression system was the first employed to recombinant protein production [Service R. F., 2002; Christendat, D *et al.*, 2000].

The most commonly used expression system is *Escherichia coli*. Protein production in bacteria offers several advantages over other hosts such as short doubling times, well-established methods for genetic manipulation, and simple, inexpensive cultivation. However, the overexpressed proteins often accumulate in insoluble and inactive deposits called inclusion bodies [Marston, F. A., 1986; King, J, *et al.*, 1996]. It has been shown that proteins tend to aggregate with increasing concentration, whereas low protein concentrations favour the formation of the correctly folded protein [Zettlmeissl, G, *et al*., 1979]. Thus, a decrease in protein synthesis rate will lead to an increase in the yield of functional protein. This was confirmed experimentally by recombinant protein expression under suboptimal conditions, i.e., the reduction of the cultivation temperature from 37 to 30°C in *E. coli* [Schein, C. H. and Noteborn, M. H. M., 1988]. However, inclusion bodies also offer some advantages, as the overexpressed protein is often highly enriched and protected from proteolytic degradation [Cheng, Y, *et al.*, 1981].

1.3.1.1 The key parameters for the *E. coli* proteins expression

1.3.1.1.1 Expression level optimization

Sometimes the levels of protein expression in the bacterial system are low despite the use of strong transcriptional and translational signals. The following approaches can be used to optimize expression levels.

The variation of induction conditions such as the time, the temperature of induction and the concentration of the inducer.

Examining the codon usage of the heterologous protein. Not all 61 mRNA codons are used equally. Each organism bears the major and the rare codons.

Usually, the frequency of the codon usage reflects the abundance of their cognate tRNAs. Therefore, when the codon usage of your target protein differs significantly from the average codon usage of the expression host, this could cause problems during expression such as mRNA instability, the premature termination of transcription and/or translation, the frameshifts, deletions and misincorporations (*e.g.* lysine for arginine) or the inhibition of protein synthesis and cell growth. As a consequence, the expression levels is often low.

Codon	*Residue*
AGG	**Arginine**
AGA	**Arginine**
CGG	**Arginine**
CGA	**Arginine**
GGA	**Glycine**
AUA	**Isoleucine**
CUA	**Leucine**
CCC	**Proline**

Table 1.3:
Favourable codon for *E. coli.*

The expressed levels can be improved by the replacing codons that are rarely found in highly expressed *E. coli* genes with more favourable codons throughout the whole gene (Table 1.3)

Moreover it is possible co-expressing the genes encoding for a number of the rare codon tRNAs. There are several commercial *E. coli* strains available that encode for a number of the rare codon genes. (Table 1.4)

Strain	*Rare codon tRNA encoded*
BL21 (DE3) CodonPlus-RIL	**arginine (AGG, AGA), isoleucine (AUA) and leucine (CUA)**
BL21 (DE3) CodonPlus-RP	**arginine (AGG, AGA) and proline (CCC)**
Rosetta	**AGG/AGA (arginine), CGG (arginine), AUA (isoleucine)**
Rosetta (DE3)	**CUA (leucine)CCC (proline), and GGA (glycine)**

Table 1.4:
***E. coli* strains that encode the rare codon tRNAs.**

Examining the second codon. The most used by *E. coli* is AAA lysine (13.9%) while a number of other codons are not used at all [Looman *et al*., 1987].

Minimizing the GC content at the 5'-end. A high GC content in the 5'-end of the gene of interest usually leads to the formation of secondary structure in the mRNA. This could result in interrupted translation and lower levels of expression. Thus, higher expression levels could be obtained by changing G and C residues at

the 5'-end of the coding sequence to A and T residues without changing the amino acids.

The addition of a a highly-expressed fusion partner often results in high level expression.

Using protease-deficient host strains (e.g. BL21) can sometimes enhance accumulation by reducing proteolytic degradation.

1.3.1.1.2 Improving protein solubility

In many cases the expressed protein is accumulated in inclusion bodies. Several strategies are available to improve the solubility of the expressed protein.

Reducing the rate of protein synthesis or changing the growth medium.

Co-expression of chaperones and/or foldases. The best characterized *E. coli* systems are: GroES-GroEL, DnaK-DnaJ-GrpE , ClpB.

Periplasmic expression. Secretion of the target protein to the periplasm has a number of distinct advantages:

- the oxidizing environment of the periplasm allows for the formation of disulfide bonds, which does not occur in the reducing environment of the cytoplasm;
- the periplasm contains two foldases, disulfide oxidoreductase (DsbA) and disulfide isomerase (DsbC), that catalyze the formation and isomerization of disulfide bonds;
- reduced proteolysis (since less proteins are present).
- allows for the accumulation of proteins that are toxic in the cytoplasm.

Secretion is achieved by the addition of a leader sequence (signal peptide) to the N-terminus of the target protein. Most used leader sequences are *pelB* and *ompT*. Unfortunately, expression yield are usually much lower and not all expressed protein is secreted into the periplasm but is also found in the medium, the cytoplasm and the cytoplasmic membrane.

Using specific host strains. Two strains are commercially available (Novagen): AD494, which has a mutation in thioredoxin reductase (trxB) and Origami, a double mutant in thioredoxin reductase (trxB) and glutathione reductase (gor). These strains have a more oxidizing cytoplasmic environment.

The addition of a soluble fusion partner often improves the solubility of the recombinant protein.

1.3.1.1.3 Expression of a protein toxic to the host strain

Low expression levels or no expression at all can also be caused by toxicity of the target protein. Many promoters are not very tightly regulated and show some degree of expression before the addition of the inducer. Different approaches can be used to give a more tightly regulated expression [Suter-Crazzolara, C. and Unsicker, K., 1995].

Constitutive expression of a protein repessing the inducible promote leaky.

Use a more tightly regulated promoter.

Use a lower copy number plasmid.

Constitutive expression of phage T7 lysosyme from a compatible pLysS or pLysE plasmid. Lysozyme binds to T7 RNA polymerase and inactivates the enzyme. After the addition of IPTG the expression level of the polymerase will be much higher than that of lysosyme and this will overcome the repression.

Use of elevated levels of antibiotics.

Use the "plating" method for inoculating cultures. Cultures are inoculated by scraping off agar plates. A single colony of plasmid-containing cells is suspended in 200 µl water and vigorously shaken. This suspension is plated on a agar plate containing the appropriate antibiotic and incubated over night at 37°C. All colonies are scrapped-of and suspended in 400 ml of medium (OD_{600}=0.4). The culture can be grown to the desired OD_{600} and induced to start expression [Suter-Crazzolara, C. and Unsicker, K., 1995].

Some proteins are so toxic for the cells that they do not only inhibit growth but also kill them. Several approaches are possible to decrease the effects of protein toxicity through the periplasmic expression (the proteins that are toxic in the cytoplasm is secreted in the periplasm or in the medium culture) or the inclusion bodies accumulation (the proteins misfolded are not toxic in the cytoplasm).

1.3.1.2 Two approaches to optimize the protein expression in *E. coli*

To produce a recombinant protein the interest gene must be inserted into an expression plasmid. This plasmid must have an replication origin (*ori*), a gene for the antibiotic resistance, an promoter to induced the protein production, a multi cloning site to insert the recombinant gene; a periplasmic signal sequence and the tags for the purification can be present.

To speed up protein production, a strategy of parallel expression of a protein from a variety of vectors containing different tags and/or fusion partners, and a variety of *E. coli* host strains can be adopted. This approach should not only gain us a lot of time but also result in a larger number of successfully expressed proteins.

1. *The expression of a protein in a basic E. coli host strain from a variety vectors with different tags and/or fusion partners.* Our first screen is to express a protein in a single host strain (e.g. BL21 (DE3)) from several vectors with different tags as fusion partners.
2. *The expression of a protein from a standard vector in a number of different E. coli host strains.* The choice of the host strains depends more on the nature of the heterologous protein.

1.3.1 EUKARIOTIC EXPRESSION SYSTEMS

To overcome the prokaryotic system limits, as the absence of post-traslational modifications or the ardous refolding protocols, the recombinant DNA technology was applied with different eukaryotic expression systems as the plant cell or transgenic plants systems, mammalian cell systems, insect cell systems and yeast cell systems. The first three systems will be treated briefly; our attention will be focused in the widening of the yeast expression system, the host cells chosen for the optimization of immunutoxin production in this work.

1.3.1.1 PROTEINS PRODUCTION IN PLANT CELL SYSTEMS

The development of methods for the introduction of foreing genes into plants has led to a revolution in plant research and plant breeding. Nowadays large areas are in use for the cultivation of such crops, including genetically modified cotton, corn and soybean [Hooykaas P.J.J., 2001], flowers with novel colours or prolonged vase life [Suzuki S., *et al.*, 2007; Yu YX., *et al.*, 2004], potatoes with optimized starch composition [Zhang L., *et al.*, 2008], rice grain with pro-vitamin A to prevent vitamin A-deficiency in the diet [Nestle M., 2001], and plants that can be used as edible vaccines [Gomez E., *et al.*, 2009; Tacket CO, 2009; Mestecky J, *et al.*, 2008]. Plant transformation relies on the totipotency of certain plant cells. In figure 1.13 several methods to create transgenic plants are shown.

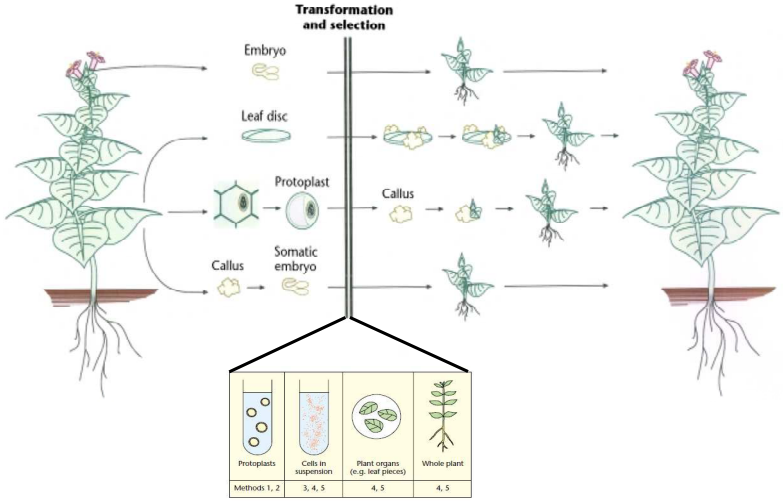


Fig. 1.13: Transformation and regeneration of plants. Many plant organs, tissues or cell types can be used for transformation. The targets for plant transformation are: 1, chemical treatment; 2, electroporation; 3, whiskers; 4, particle bombardment; 5, *Agrobacterium.* [Modified by Crouzet P. and Hohn B., 2002; Hooykaas P.J.J., 2001]

After plant cells have been transformed, it is essential that the transformed cells can be recognized, isolated or specifically propagated through the bacterial antibiotic resistance genes converte into plant-expressed forms or through the

genes that confer tolerance at the herbicides or thank at the visible markers as the β-glucuronidase (GUS) gene [Jefferson *et al*., 1987], the firefly luciferase genes [Ow *et al*., 1986] and the jelly fish green fluorescent protein (GFP) [Haseloff *et al*., 1997].

Plants are an attractive host for synthesis of many industrial and pharmaceutical proteins, offering significant advantages in safety and cost over alternative foreign protein expression systems [Twyman *et al.,* 2003; Ma *et al.,* 2005; Streatfield, 2007]. The main advantages associated with plants include the low cost of agricultural biomass production, elimination of downstream processing requirements for vaccines expressed in edible plant tissues, post-translational modification and production of correctly folded and assembled multimeric proteins, low risk of contamination with pathogens and endotoxins such as those occurring in mammalian and bacterial systems, and the avoidance of ethical problems associated with transgenic animals and animal materials.

Table 1.5 show an example of foreign protein produced in whole plants.

The products that are currently being produced in plants include bioactive peptides, vaccine antigens, antibodies, diagnostic proteins, nutritional supplements, enzymes and biodegradable plastics. The economic driver that is burgeoning this industry includes the ability to synthesize animal proteins, cost benefits, safety issues (lack of pathogen contamination) and easy scale up [Fischer *et al.,* 2004; Lal *et al*., 2007; Rybicki, 2009; Sharma *et al*., 2004; Spok, 2007; Stoger *et al.,* 2002; Twyman *et al.,* 2003]. Apart from many advantages, there are some problems associated with plants for their use as bioreactors which include differences in glycosylation patterns in plants and humans, inefficient expression and environmental contamination. However the the transgenic plant-based products commercially available in the market and the products in phase II / I or pre-clinical trials were produced successfully (Tables 1.6 and 1.7) but none of these is an Immunotoxin.

Vector	Recombinant protein	Production system	Significant result	Expression	Reference
TMV	Single-chain of bFSH (sc-bFSH)	*Nicotiana benthamiana*	Expressed native-like structure of sc-bFSH, similar N-glycosylation to mammalian origin, *in vivo* bioactivity	sc-bFSH: 3% of TSP	Dirnberger et al. (2001)
TMV	Murine zona pellucida (ZP) 3 protein	*Nicotiana tabacum*	Generated immune response in mice	Virus: 2.5 mg g^{-1} leaf	Fitchen et al. (1995)
TMV	Core neutralizing epitope (COE) of PEDV	*Nicotiana tabacum* cv. Havana	Recombinant COE gene with modified codon usage pattern and removal of mRNA destabilising motifs gave a protein yield >30 times higher than the native COE gene	Recombinant COE protein: 130 μg g^{-1} leaf or 5% of TSP	Kang et al. (2004)
TMV	scFv of immunoglobulin from 38C13 mouse B cell lymphoma	*Nicotiana benthamiana*	Generated effective immune response and immunoprotection against tumour challenge in mice	scFv protein: 12.3–30.2 μg g^{-1} leaf	McCormick et al. (1999)
TMV	BHV-1 protein glycoprotein D (gDc)	*Nicotiana benthamiana*	Generated effective immune response and protection against viral challenge in animal models	gDc: 20 μg g^{-1} leaf	Pérez Filgueira et al. (2003)
TMV	Structural protein VP1 of FMDV and HIV-1 p24 nucleocapsid protein	*Nicotiana benthamiana*	Generated effective immune response in rabbits, native antigenic properties were mostly preserved	VP1: 6–8 μg g^{-1} leaf p24: 0.1 mg g^{-1} leaf	Pérez-Filgueira et al. (2004)
TMV	Antigenic peptides from influenza virus HA and HIV-1	*Nicotiana tabacum* cv. Samsun	Similar yield of the virus particles to wild-type; foreign peptides of 21 amino acids were presented on the particle surface of TMV	Virus: 0.8–2 mg g^{-1} leaf	Sugiyama et al. (1995)
TMV	Structural protein VP1 of FMDV	*Nicotiana benthamiana*	Generated effective immune response and protection against viral challenge	VP1: 50–150 μg g^{-1} leaf	Wigdorovitz et al. (1999)
TMV	Two immunogenomic dominant epitopes of FMDV serotype O (TMVF11 and TMVF14)	*Nicotiana tabacum* Xanthi	Generated effective immune response and protection against viral challenge in most immunised animal models	TMVF11 or TMVF14: 5 mg g^{-1} leaf	Wu et al. (2003)
PVX	HBV nucleocapsid protein (HbcAg)	*Nicotiana benthamiana*	More than five-fold higher HBcAg yield using transient rather than stable expression using CPMV in *Vigna unguiculata*	HBcAg: >50 μg g^{-1} leaf	Mechtcheriakova et al. (2006)
PVX	scFv against herbicide diuron	*Nicotiana benthamiana*	PVX vector used as a presentation system for expressing scFv	Virus: 200–500 μg g^{-1} leaf scFv-CP fusion protein: 100–250 μg g^{-1} leaf (4 dpi)	Smolenska et al. (1998)
CPMV	Epitopes derived from MEV	*Vigna unguiculata* var blackeye	Generated protection against viral challenge	Virus: 1–1.2 mg g^{-1} leaf	Dalsgaard et al. (1997)
CPMV	Epitopes derived from HRV-14 and HIV-1	*Vigna unguiculata* var blackeye	Exhibited the antigenic properties of inserted sequence; generated effective immune response in rabbits when immunised with HRV-14 epitope containing virus	Virus: 1.2–1.5 mg g^{-1} leaf	Porta et al. (1994)
ZYMY	GFP and *Dermatophagoides pteronyssinus* group 5 allergen (Der p 5) with histidine tag	*Cucurbita pepo* L var Zucchini (squash)	Inhibited synthesis of specific IgE and airway inflammation in animal studies immunised with Der p 5	GFP: 3.7 μg g^{-1} leaf Der p 5: 1.5 μg g^{-1} leaf (8–10 dpi)	Hsu et al. (2004)
ORSV and TMV	Neuropeptide nocistatin	*Nicotiana benthamiana* and *N. tabacum* cv. Turkish, respectively	Complete peptide without additional amino acid sequence was expressed	ORSV-mNST: 2.05 μg g^{-1} leaf TMV-mNST: 5.2 μg g^{-1} leaf (14 dpi)	Lim et al. (2002)
TBSV	HIV-1 p24 nucleocapsid protein	*Nicotiana benthamiana*	Bound specifically to anti-p24 antibody, confirming maintenance of antigenic determinants	p24 fusion protein: 5% of soluble leaf protein	Zhang et al. (2000)

Abbreviations: bFSH, bovine follicle stimulating hormone; BHV-1, bovine herpes virus type 1; CP, coat protein; CPMV, cowpea mosaic virus; dpi, days post-inoculation; FMDV, foot-and-mouth disease virus; GFP, green fluorescent protein; HBV, hepatitis B virus; HIV-1, human immunodeficiency virus type 1; HRV-14, human rhinovirus type 14; IgE, immunoglobulin E; MEV, mink enteritis virus; ORSV, odontoglossum ringspot virus; PEDV, porcine epidemic diarrhea virus; PVX, potato virus X; scFv, single-chain variable fragment; TBSV, tomato bush stunt virus; TMV, tobacco mosaic virus; TSP, total soluble protein; ZYMY, zucchini yellow mosaic virus.

Table 1.5: Example of foreign proteins produced in whole plants using plant viral vectors.
[Modified by Sharma A. K., Sharma M. K .,2009]

Product name	Company name	Plant system	Commercial name	Catalog no./URL
Avidin	Prodigene	Corn	Avidin	#A8706, Sigma-Aldrich
β-Glucuronidase	Prodigene	Corn	GUS	#G2035, Sigma-Aldrich
Trypsin	Prodigene	Corn	TrypZean™	#T3568, Sigma-Aldrich
Recombinant human lactoferrin	Meristem Therapeutics, Ventria Bioscience	Corn, rice	Lacromin™	#L4040, Sigma-Aldrich
Recombinant human lysozyme	Ventria Bioscience	Rice	Lysobac™	#L1667, Sigma-Aldrich
Aprotonin	Prodigene	Corn, transgenic tobacco	AproliZean	#A6103, Sigma-Aldrich
Recombinant lipase	Meristem Therapeutics	Corn	Merispase®	http://www.meristem-therapeutics.com[a]
Recombinant human intrinsic factor	Cobento Biotech AS	*Arabidopsis*	Coban	http://www.cobento.dk/?id=76
Vaccine purification antibody	CIGB, Cuba	Tobacco	–	Kaiser, 2008

[a] MERISTEM Therapeutics & SBH Sciences has announced an agreement for joint development of animal-free recombinant proteins.

Table 1.6: Transgenic plants-based products commercially available in the market
[Modified by Sharma A. K., Sharma M. K .,2009]

Product name	Company/institute name	Plant system	Reference/URL
Human serum albumin (HSA)	Chlologen Inc.	Tobacco	Currently technology is with Dow AgroSciences
38C13 (scFv)	Large Scale Biology Corp.	Tobacco	http://www.lsbc.com[a]
RhinoX™	Planet Biotechnology Inc.	Tobacco	http://www.planetbiotechnology.com/
Alpha inteferon 2b	Biolex Therapeutics Inc.	*Lemna*	http://www.biolex.com/
DoxoRX™	Planet Biotechnology Inc.	Tobacco	http://www.planetbiotechnology.com/
α-Caries MAb (CaroX™)	Planet Biotechnology Inc.	Tobacco	http://www.planetbiotechnology.com/
Aprotonin	Large Scale Biology Corp.	Tobacco	http://www.lsbc.com[a]
Collagen	Medicago Inc.	Alfalfa	http://www2.medicago.com/en/product/
	Meristem Therapeutics	Tobacco	http://www.meristem-therapeutics.com[b]
Lipase	Meristem Therapeutics	Maize	http://www.meristem-therapeutics.com[b]
Lactoferrin	Meristem Therapeutics	Tobacco	http://www.meristem-therapeutics.com[b]
	Ventria Bioscience	Rice, maize	http://www.ventriabio.com/
Lysozyme	Ventria Bioscience	Rice	http://www.ventria.com/;
BLX-301 (anti-CD antibody)	Biolex Therapeutics Inc.	*Lemna*	http://www.biolex.com/blx301.htm
GLA rich safflower oil	Sembiosys Genetics Inc.	Safflower	http://www.sembiosys.com/
DHA rich safflower oil	Sembiosys Genetics Inc.	Safflower	http://www.sembiosys.com/
Human intrinsic factor	Cobento Biotech AS	*Arabidopsis*	http://www.cobento.com/
Human glucocerebrosidase	Protalix Biotherapeutics	Carrot suspension cells	http://www.protalix.com/glucocerebrosidase.html
Parvovirus vaccine	Large Scale Biology Corp.	*Nicotiana*	http://www.lsbc.com[a]
Anti-F4 fimbriae of ETEC antibody	Novaplant	Pea (field trials completed 2007)	http://www.novoplant.de/fileadmin/redaktion/seitenspezifische_inhalte/news/pr_FieldTrial_210907.pdf
Protein A reagent (Stratocapture™)	Sembiosys Genetics Inc.	Safflower	http://www.sembiosys.com/

[a] LSBC filed bankruptcy in 2006.
[b] Meristem Therapeutics & SBH Sciences has announced an agreement for joint development of animal-free recombinant proteins.

Table 1.7: List of the products in phase II, I or preclinical trials.
[Modified by Sharma A. K., Sharma M. K .,2009]

1.3.1.2 PROTEINS PRODUCTION IN MAMMALIAN CELLS

Cultivated mammalian cells have become the dominant system for the production of recombinant proteins for clinical applications because of their capacity for proper protein folding, assembly and post-translational modification.

In 1986 human tissue plasminogen activator (tPA, Activase; Genentech, S. San Francisco, CA, USA) became the first therapeutic protein from recombinant mammalian cells to obtain market approval. The recombinant proteins are expressed in immortalized Chinese hamster ovary (CHO) cells, cell lines derived from mouse myeloma (NS0), baby hamster kidney (BHK), human embryo kidney (HEK-293) and human retinal cells. The development of a manufacturing process for a recombinant protein in mammalian cells usually follows a well-established scheme (Fig. 1.14).

Initially, the recombinant gene with the necessary transcriptional regulatory elements is transferred to the cells [Ringold, G., *et al.*, 1981; Gopalkrishnan, R.V. *et al*., 1999]. In addition, a second gene is transferred that confers to recipient cells a selective advantage. In the presence of the selection agent, which is applied a few days after gene transfer, only those cells that express the selector gene survive [Balland, A. *et al*, 1988]. The most popular genes for selection are dihydrofolate reductase (DHFR), an enzyme involved in nucleotide metabolism, and glutamine synthetase (GS).

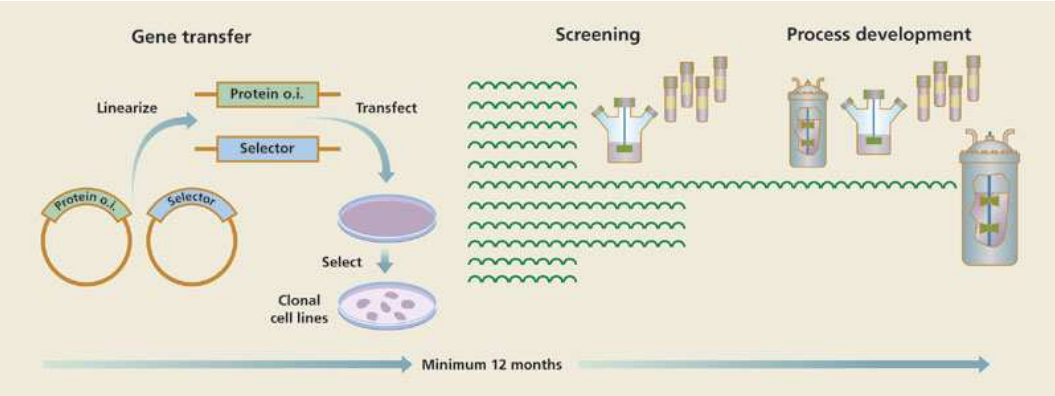


Fig 1.14: Cell line generation and development for cell culture processes for the generation of recombinant proteins of interest (o.i.). The wavy lines indicate subcultivations of individual cell lines that are in a screening program to obtain the final producer. Vials indicate banks of cells frozen in liquid nitrogen. Spinner flasks represent scale-down systems for process optimization, and bioreactors represent large-scale production processes. [Modified by Wurm F M., 2004]

Following selection, survivors are transferred as single cells to a second cultivation vessel, and the cultures are expanded to produce clonal populations

[Barnes, L.M., 2004; Jones, D. *et al.*, 2003]. Eventually, individual clones are evaluated for recombinant protein expression, with the highest producers being retained for further cultivation and analysis. From these candidates, one cell line with the appropriate growth and productivity characteristics is chosen for production of the recombinant protein.

Although the basic concepts have not changed since the mid-1980s, the productivity of recombinant cell lines has increased dramatically in the past two decades. Overall, efforts have led to improvements in vectors, host cell engineering, medium development, screening methods and process engineering and development [Wurm F M., 2004].

Two main formats have been employed for the large scale production of recombinant proteins in mammalians cells: cultures of adherent cells [Loumaye, E. *et al*. 1998; Spier, R. & Kadouri, A., 1997] and suspension cultures [Barnes, L.M., *et al*. 2000; Bödecker, B.G.D., *et al*. 1994; Wurm, F.M. & Bernard, A.R., 1999; Jones, D. *et al*., 2003]. The latter is by far the most common.

1.3.1.2.1 Immunotoxins produced with mammalian cell systems

In the table 1.8, some Immunotoxins produced in the mammalian cell system are summarized.

Immunotoxin	***Description***	***Reference***
ImmunoRNase	The IT is expressed in myeloma cells.	Krauss J, *et al.,* 2009
VEGF165-PE38	Vascular Endothelial Growth Factor 165 fused to PE38.	
	The IT is expressed to mesenchymal stem cells that were used as a vehicles for delivery the drug.	Hu CC, *et al.,* 2009
	The IT is expressed in HEK293 cells.	Hu CC, *et al.,* 2010
Gb-H22 (scFv)	Granzyme B fused to the scFv anti-CD64. The IT is expressed in human 293T cells	Stahnke B, *et al.,* 2008
Ki4-angiogenin C-term **Ki4-angiogenin N-term**	The anti-CD30 scFv (Ki4) fused to the C- and N- terminal of angiogenin. These ITs are expressed in human 293T cells	Stöcker M., *et al.,* 2003

Table 1.8: Some Immunotoxins produced in the mammalian cell systems.

1.3.1.3 PROTEINS PRODUCTION IN INSECT CELLS

The introduction of the functional expression of recombinant proteins, the use of baculovirus vectors to produce proteins for purification has become one of the most widely-used viral gene delivery systems. Extensive engineering to simplify and accelerate the process of recombinant virus construction has made this system accessible to virtually any modern biological laboratory.

Viruses are a natural choice for exploitation as gene transfer vectors due to their inherent ability to package nucleic acids and deliver them to susceptible cells. Because of several unique features, such as extremely high levels of gene expression, accessibility, and an excellent biosafety profile, baculoviruses have been one of the most popular viral vectors for expression of recombinant proteins since their introduction in 1983. The majority of commercially-available baculovirus vector systems are based on the budded form of the *Autographa californica* multiple nucleopolyhedrosis virus (AcMNPV) [Funk, C.J., *et al.*, 1997]. Baculovirus vectors are generally propagated in cells originally isolated from *Spodoptera frugiperda* (Sf9 or Sf21) [O'Reilly, D.R., *et al.*, 1992]. These cells are commonly used for production of recombinant proteins such as enzymes, nuclear receptors, G protein-coupled receptors, ion channels, cytochrome P450s, secreted proteins, etc. that have been expressed in a functional form.

Insect cell over-expression has the advantage over bacterial expression that since the cells are eukaryotic post-translational modifications of a protein are in many respects identical to those carried out in mammalian cells [Jarvis, D.L., 1997]. The major difference between proteins produced in insect and mammalian cells is in the composition of carbohydrate modifications added post-translationally [Kost, T.A, *et al.*, 2005].

To construct a baculovirus vector the recombinant gene expression cassette must be inserted into the viral genome. In its original conformation (Fig. 1.15A) [Smith, G.E., *et al.*, 1983; Pennock, G.D., *et al.*, 1984] this was accomplished by subcloning the viral polyhedrin protein gene locus into a plasmid vector and inserting the gene of interest into the polyhedrin gene such that its expression is controlled by this highly active late viral promoter. This plasmid is transfected into insect cells along with baculovirus DNA and homologous

recombination is allowed to bring the recombinant gene into the viral DNA [Smith, G.E., *et al.*, 1983; Pennock, G.D., *et al.*, 1984].

In figure 1.15 four major themes in construction of recombinant viral genomes are illustrated.

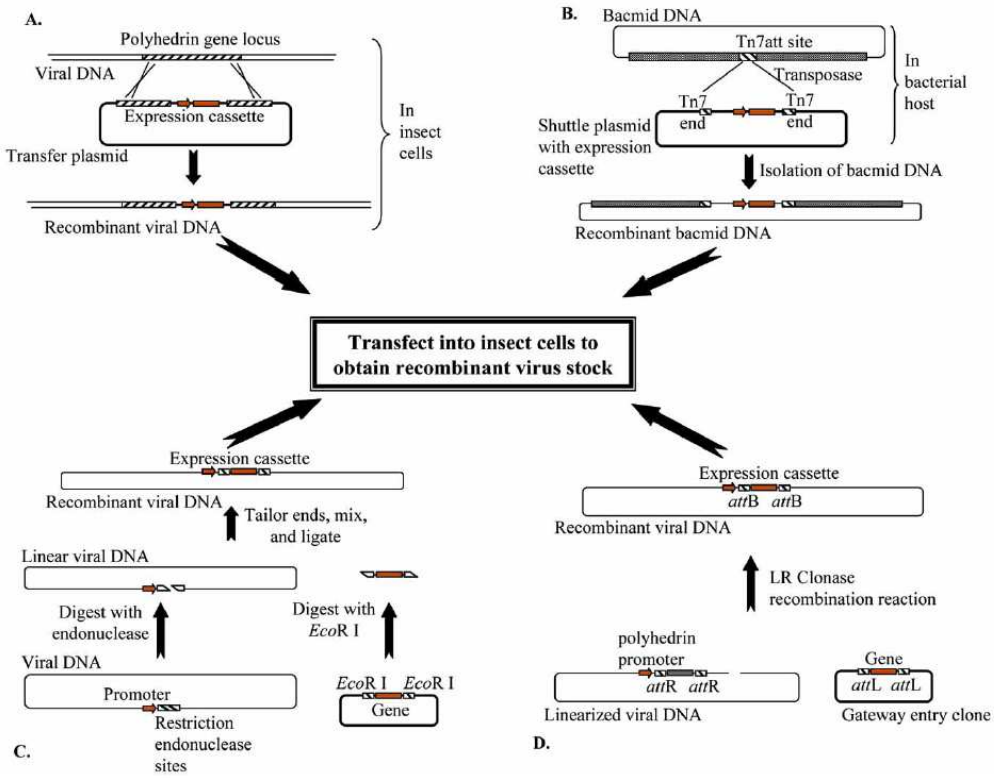


Fig. 1.15: Methods of insertion of transgene expression cassettes for construction of recombinant baculovirus genome. (**A**) Homologous recombinantion in insect cells [Kitts, P.A., *et al.*, 1990; Kitts, P.A. and Possee, R.D., 1993]. A commercially-available system was developed by Oxford Expression Technologies and is termed flash- BAC™. (**B**) Site-specific transposition in bacterial cells [Luckow, V.A., *et al.*, 1993]. This system has been commercialized under the name Bac-to-Bac™ by Invitrogen. (**C**) Directed *in vitro* cloning of restriction fragments into viral DNA [Ernst, W.J., *et al.*, 1994; Lu, A. and Miller, L.K., 1996]. A commercially-available system was developed by Invitrogen. (**D**) Site-specific recombination *in vitro*.[Modified by J. P. Condreay and T. A. Kost. , 2007].

In addition to its role as an insect cell protein expression system, baculovirus mediated gene delivery into mammalian cells has recently evolved into a particularly useful research tool [Kost, T.A., *et al.*, 2005; Hofmann C, *et al.*, 1995; Boyce, F.M. and Bucher, N.L., 1996].

In summary, the versatile baculovirus system continues to be widely used in pharmaceutical discovery research programs. The applications of this powerful viral vector system will evolve with further advances in the understanding of baculovirus biology and the interaction of modified viruses with mammalian cells.

1.3.1.3.1 Immunotoxin produced with insect cell system

In the table 1.9 two Immunotoxins produced in the insect cell system are reported.

Immunotoxin	*Description*	*Reference*
scFv-mel-FLAG	Recombinant melittin-based cytolytic Immunotoxin	Choo AB., *et al.,* 2002
DTctGMCSF	The human granulocyte-macrophage colony stimulating factor (GMCSF) fused by truncated form of DT lacking the binding domain.	Williams MD., *et al.,*1998

Table 1.9: Immunotoxins produced in the insect cell systems.

1.3.1.4 PROTEINS PRODUCTION IN YEAST SYSTEMS

Yeasts are among the earliest-domesticated microorganisms. They are unicellular, eukaryotic fungi, the cells having a membrane-bounded nucleus and membrane bounded mitochondria. Yeasts occur in many habitats, usually associated with numerous other living things. There are approximately 700 known yeast species in 100 genera [Spencer A.I.R and Spencer J.F.T, 2002]. Most of the ascomycetous yeasts are in the genus *Pichia* (91species), and the nonsporulating forms in *Candida* (163 species), *Cryptococcus* and *Rhodotorula* (34 species each) (Kurtzman and Fell, 1998).

Humans have cultivated yeast since the dawn of agriculture to make beer, bread, and wine. As a domesticated microorganism and sexual eukaryote, the budding yeast *Saccharomyces cerevisiae* is the most commonly used industrial yeast and has emerged as a remarkably tractable eukaryotic model system. Other widely studied unicellular fungi, particularly the fission yeast *Schizosaccaromyces pombe* and the orange bread mould *Neurospora crassa*, share many of the traits that make *S. cerevisiae* attractive to investigate and are useful for analysing numerous genetic phenomena with no parallel in the budding yeast. Yeasts belong to the kingdom of fungi, along with moulds, smuts, and mushrooms. They share a common cellular architecture and rudimentary life cycle with multicellullar eukaryotes such as plants and animals [Mell J.C. and Burgess S.M., 2002]. As nonpathogenic, nonmotile microorganisms, yeasts are easily propagated and manipulated in the laboratory. An important theme of yeast biology is the use and study of homologous recombination, a process by which a broken piece of DNA uses a homologous DNA template as a substrate for repair. Yeast use this process to fix DNA damage, to switch mating types, and to segregate homologous chromosomes during meiosis.

Like all eukaryotes, yeast cells have numerous membrane bound organelles, including a nucleus, endosymbiotic mitochondria, the peroxisome, and the organelles of the secretory pathway. The budding yeast carries its genome of nearly 6000 genes in 12 megabase pairs of DNA on 16 linear chromosomes in the nucleus. The genome is very compact for a eukaryote: the number and size of genes are relatively small and the density of genes is relatively high. Genetic redundancy in the yeast genome is low, facilitating the analysis of gene function.

The life cycle of the budding yeast is rudimentarily similar to that of any sexual eukaryote, alternating between haploid and diploid states, containing one and two sets of chromosomal complements, respectively. Cells divide by budding; a mother cell buds to produce a genetically identical daughter cell. Before the daughter is released, copies of each chromosome (called sister chromatids) segregate by mitosis. In a haploid, there is one of each chromosome in the complement. When two haploid cells mate and fuse, they yield a diploid cell, which contains two of each chromosome. These pairs are called homologous chromosomes. A diploid can either grow by budding or undergo meiosis, a double round of cell division yielding four haploid spores held together in an ascus. As in other eukaryotes, yeast cells age. A mother cell produces a limited number of daughter cells. Life span in yeast is under the control of a genetic programme that shares features in common with multicellular animals [Mell J.C. and Burgess S.M., 2002].

1.3.1.4.1 STEPS FOR THE RECOMBINANT PROTEINS PRODUCTION IN THE YEAST SYSTEMS

The protein expression in *Escherichia coli* or in yeast such as *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* has been preferred for investigations where a high yield of expressed functional protein is the main goal. Among the various yeast expression systems used thus far for the production of foreign proteins, the baker's yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* account for the most commonly used forms. The choice of one over the other or even the selection of other yeasts like *Pichia pastoris, Hansenula polymorpha, Kluyveromyces lactis, Yarrowia lipolitica* or *Candida utilis* will depend on the scope of the investigation. Irrespective of the host used, successful expression includes the introduction of the foreign DNA into the yeast cells, the maintenance of the foreign DNA in the host, and the assurance of efficient expression.

1.3.1.4.1.1 Introduction of foreign DNA and its stability in yeast cells

The genetic transformation of yeast was first made possible by the development of methods in which foreign DNA, in the presence of calcium and

polyethylene glycol, was introduced into spheroplasts produced by enzymatic removal of the cell wall. Meanwhile, various newer, time and transformation-efficient methods, like the lithium acetate method [Ito *et al.*, 1983], or electroporation techniques [Becker and Guarente, 1991], have also ensured the successful transfer of DNA into host cells that retain an intact cell wall.

Successful translation of foreign DNA in yeast depends on the vectors used as well as the species or strain of host cells [Kingsman *et al.*, 1985; Stearns *et al.*, 1990; Rose and Broach, 1990].

For selective growth in yeast, all plasmids are equipped with selectable markers – yeast genes that complement specific auxotrophies of the host strain used. Thus, yeast *leu2*$^{-}$ mutants can grow in media lacking leucine only when they are transformed with plasmids bearing the LEU2 gene. The other most commonly used selectable markers are HIS3, URA3, TRP1 or LYS2 genes, which confer auxotrophy to strains incapable of synthesizing histidine, uracil, tryptophan or lysine, respectively.

1.3.1.4.1.2 Efficient expression

Efficient transcription can be achieved by the promoter and terminator sequences that flank the foreign DNA. A series of efficient promoters for expression in *S. cerevisiae*, such as the GAL4, ADH, PGK or GAPDH promoters, are derived from the 5' flanking regions of glycolytic genes of this organism. Terminator regions are fragments often derived from the PGK, PHO5 or TRP1 genes and are in general not as well characterized as promoters. Careful consideration of all these factors, however, does not necessarily guarantee successful expression of the foreign protein. In some cases, instability is due to degradation by yeast endogenous proteases, and the use of yeast strains deficient in some of the proteases might help to overcome the problem.

Yeast cells have been used for the production of various proteins of clinical or other commercial interest. In many cases, proteins are produced by using a secretion system involving the prepro-a-factor leader region of *S. cerevisiae* [Zsebo *et al.*, 1986; Wood *et al.*, 1985; Bitter *et al.*, 1988; Barr *et al.*, 1988].

1.3.1.4.2 Recombinant protein production with the *Pichia pastoris* expression system

The production of a functional protein is intimately related to the cellular machinery of the organism producing the protein. The yeast *Pichia pastoris* is a useful system for the expression of milligram-to-gram quantities of proteins for both basic laboratory research and industrial manufacture. The fermentation can be readily scaled up to meet greater demands, and parameters influencing protein productivity and activity, such as pH, aeration and carbon source feed rate, can be controlled [Higgins DR and Cregg JM , 1998]. Compared with mammalian cells, *Pichia* does not require a complex growth medium or culture conditions, is genetically relatively easy to manipulate, and has a eukaryotic protein synthesis pathway. Some proteins, such as G protein-coupled receptors, that cannot be expressed efficiently in bacteria, *Saccharomyces cerevisiae* or the insect cell/baculovirus system, have been successfully produced in functionally active form in *P. pastoris* [Cereghino GPL, *et al* 2001; Cereghino GPL, *et al.*, 2002; de Jong LAA, *et al.*, 2004]. This methylotrophic yeast is particularly suited to foreign protein expression for a number of reasons, including ease of genetic manipulation, e.g. gene targeting, high-frequency DNA transformation, cloning by functional complementation, high levels of protein expression at the intra- or extracellular level, and the ability to perform higher eukaryotic protein modifications, such as glycosylation, disulphide bond formation and proteolytic processing [Cregg JM, *et al.,* 2000]. *Pichia* can be grown to very high cell densities using minimal media [Veenhuis M, *et al.,* 1983; Wegner G., 1990] and integrated vectors help genetic stability of the recombinant elements, even in continuous and large scale fermentation processes [Romanos M., 1995]. Simple purification of secreted recombinant proteins is possible due to the relatively low levels of native secreted proteins [Cregg JM, *et al.*, 1993]. Therefore, the powerful genetic techniques available, together with its economy of use, make *P. pastoris* a system of choice for heterologous protein expression.

1.3.1.4.2.1 The *Pichia pastoris* history

The Phillips Petroleum Company was the first to develop media and protocols for growing *P. pastoris* on methanol in continuous culture at high cell densities (>130 g/dry cell weight [Cereghino JL and Cregg JM. 2000]). During the 1970s, *P. pastoris* was evaluated as a potential source of single-cell protein due to the ability of this yeast to utilize methanol as sole carbon source. Unfortunately, the oil crisis of the 1970s caused a dramatic increase in the cost of methane (the source of the methanol). Simultaneously, the price of soybeans, the major alternative source of animal feed, fell. However, in the following decade, Phillips Petroleum, together with the Salk Institute Biotechnology/ Industrial Associates Inc. (SIBIA, La Jolla, CA, USA), studied *P. pastoris* as a system for heterologous protein expression. The gene and promoter for alcohol oxidase were isolated by SIBIA, who also generated vectors, strains and corresponding protocols for the molecular manipulation of *P. pastoris*. What began more than 20 years ago as a program to convert abundant methanol to a protein source for animal feed has developed into what are today two important biological tools: a model eukaryote used in cell biology research, and a recombinant protein production system. *Pichia* has gained widespread attention as an expression system because of its ability to express high levels of heterologous proteins. As a result, recombinant vector construction, methods for transformation, selectable marker generation, and fermentation methods have been developed to exploit the productive potential of this system [Rosenfeld SA, *et al.,* 1999]. Research Corporation Technologies (Tucson, AZ, USA) are the current holders of the patent for the *P. pastoris* expression system, which they have held since 1993, and the *P. pastoris* expression system is available in kit form from Invitrogen Corporation (Carlsbad, CA, USA).

1.3.1.4.2.2 Comparison between *E. coli* and *P. pastoris* expression systems

Over the past three decades, *Escherichia coli* has been extensively used as a cellular host for protein expression. However, the broad application of this system with proteins derived from eukaryotic genomes that require post-translational modifications has been problematic. Successful expression of

recombinant proteins in *E. coli* is thus very dependent on the primary, secondary, tertiary and functional characteristics of the protein of interest. As *E. coli* is a prokaryote, the inability to correctly fold the foreign protein and perform other post-translational modifications limits the types of proteins that can be expressed. Since the protein product may be typically obtained as insoluble, miss-folded inclusion bodies, subsequent solubilization and re-folding steps are required and the folding conditions need to be carefully optimized [Makrides SC., 1996; Marston FA. 1986; Majerle A, *et al.,* 1999; Chen BP and Hai T. 1994; Koganesawa N, *et al.,* 2001] . Development of such folding conditions may be difficult to achieve and time consuming [Wang P, *et al.,* 2000; Tsujikawa M, *et al.,* 1996] and these factors can result in significant losses, lower productivities and increased costs of manufacture of the expressed protein. Moreover *E. coli* expressed proteins also tend to retain their amino-terminal methionine, which may affect protein stability [Chaudhuri TK, *et al.,* 1999; Takano K, *et al.,* 1999] and cause immunogenicity. Since *E. coli* has no capacity to glycosylate recombinant proteins, this constraint is an important factor that can alter the function of certain proteins [Meldgaard M, *et al.,* 1994; Jenkins N, *et al.,* 1996].

A large variety of the proteins that cannot be expressed in *E. coli* at the correct level of post-translational maturation have subsequently been produced in the methylotrophic yeast, *Pichia pastoris* [Monsalve RI, *et al.,* 1999]. For example, this trend can been exemplified from the comparative studies by Lueking et al. [Lueking A, *et al.,* 2000], where expression vectors for *E. coli* and *P. pastoris* were constructed and tested by cloning various cDNAs from a human fetal brain expression library. From the 29 different cDNA clones that were in the correct reading frame, all were found to produce soluble proteins in *P. pastoris*. With the *E. coli* expression system on the other hand, only nine clones resulted in soluble proteins, 15 were detected as inclusion bodies and five were not expressed at all. This outcome is undoubtedly due to differences in the protein folding environment and the inability of *E. coli* to perform post-translational modifications [Lueking A, *et al.,* 2000]. Other proteins, generated as mis-folded, insoluble inclusion bodies through expression in *E. coli* but obtained as soluble and correctly folded proteins when expressed in *P. pastoris*, include antigen 5 (Ag5) from the yellow-jacket, paper-wasp and white-face hornet wasp species

[Monsalve RI, *et al.,* 1999; King Te P, *et al.,* 1995], herring antifreeze protein [Li ZJ, *et al.,* 2001; Ewart KV, *et al.,* 1996], prourokinase [Holmes WE, *et al.,* 1985] and human transferring [Hershberger CL, *et al.,* 1991; Mason AB, *et al.,* 1996].

P. pastoris expression systems therefore offer significant advantages over *E. coli* expression systems for the production of many heterologous eukaryotic proteins. As noted above, this feature is particularly relevant for target proteins containing multiple disulphide bonds or requiring glycosylation, phosphorylation, the absence of an amino-terminal methionine or no oligomer formation for the correct assembly of the mature protein. *P. pastoris* expression systems, besides permitting the authentic protein to be secreted in soluble form, also has the capability of producing large quantities of the recombinant protein. Developments with this system include the choice of various *P. pastoris* strains and vectors that enable a wide choice of promoters, secretion signals and selectable markers to be made.

1.3.1.4.2.3 *P. pastoris* EXPRESSION STRAINS

Numerous strains of *P. pastoris* with a wide range of genotypes are now available. The choice of a specific strain is determined by the required application. The genotype and phenotype characteristics of a number of the more useful strains are summarized in table 1.10.

Strain	Genotype	Phenotype	Reference
SMD1168	*His4, pep4*	Mut^+, His^-, $pep4^-$	White CE, et al., 1995
GS115	*his4*	Mut^+, His^-	Cregg JM et al., 1985
KM71	*His4, aox1:ARG4, arg4*	Mut^S, His^-,	
X-33	Wild type	-	Li ZJ et al., 2001
MP-36	-	-	Paifer E, et al., 1994
SMD1165	*His4, prb1*	Mut^+, His^-, prb^-	Abdulaev NG et al., 1997
SMD1163	*his4, prb1, pep4*	Mut^+, His^-, $pep4^-$, prb^-	Sreekrishna K, et al., 1997
MC100-3	*Arg4 his4 aox1 Δ:: SARG4 aox2Δ:: Phis4*	Mut^-, His^-,	Gellissen G., 2000 Inan M and Meagher MM. 2001

Table 1.10: Genotype and phenotype of *P. pastoris* strains.
[Modified by Daly R. and Hearn M.T.W., 2005]

The SMD1168 and SMD1168H strains, for example, are defective in the vacuole peptidase A (pep4). This enzyme is responsible for activating carboxypeptidase Y and protease B1 and, hence, these SMD1168 and SMD1168H strains are also defective in these proteases [White CE, *et al.*, 1994]. Strains such as KM71, GS115 and SMD1168 are defective in the histidine dehydrogenase gene (his4). Their use allows transformants to be selected based on their ability to grow in non-histidine containing media. The strains SMD1168 and GS115 both contain a functional copy of the alcohol oxidase 1 gene (AOX1) responsible for approximately 85% of the utilization of methanol by the alcohol oxidase enzyme. These strains therefore have a wild-type methanol utilization phenotype designated as Mut^+ [Inan M and Meagher MM. 2001]. The KM71 strain, however, contains a non-functional AOX1 (aox1) and relies on the alcohol oxidase enzyme being produced from an alternative gene called AOX2. The AOX2 enzyme has the same specific activity as AOX1 but has a much lower expression level (weaker promoter) and can only consume methanol slowly, hence the phenotype of these strains are termed 'methanol utilization slow' (Mut^S) [Inan M and Meagher MM. 2001; Cregg JM, , *et al.*, 1993].

1.3.1.4.2.4 AOX pathways

Methylotrophic yeasts such as *P. pastoris* can use methanol as a sole carbon source. The methanol is oxidised to formaldehyde by alcohol oxidase in the peroxisome (Fig. 1.16) [Tschopp JF, *et al.*, 1987; Cereghino JL and Cregg JM., 2000; Couderc R and Baratti J. 1980].

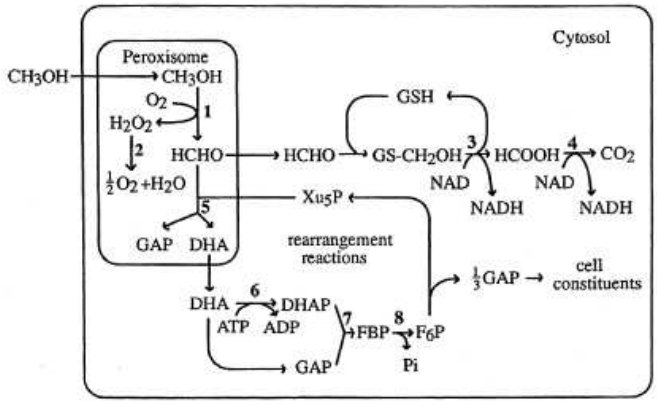


Fig 1.16: The methanol pathway in *P. pastoris.*
1. alcohol oxidase; 2. catalase; 3. foemaldehyde dehydrogenase; 4. formate dehydrogenase; 5. dihydroxyacetone synthase; 6. dihydroxyacetone kinase; 7. fructose 1,6-biphosphate aldolase; 8. fructose 1,6-bisphosphatase. [Modified by Cereghino J.L., Cregg J.M. 2000].

Alcohol oxidase is an oligomeric flavo-protein and contains an FAD and the analogue

mFAD non-covalently attached to accomplish electron transfer. AOX is strongly repressed by many alternate carbon sources such as glucose and glycerol and is induced by carbon starvation and methanol. When *P. pastoris* cultures are grown on methanol as a carbon source, it has been shown that the AOX mRNA can comprise up to 5% of the total polyA-RNA pool [Cregg JM and Higgins DR. 1995]. The reaction catalysed by AOX requires O_2 and forms H_2O_2 and formaldehyde. The alcohol oxidase is not specific for methanol and can oxidize other primary alcohols; however, as the number of carbons in the alcohol increases, the activity of the enzyme decreases. The H_2O_2 produced by the AOX reaction is converted to H_2O by peroxisome catalase [Gellissen G. 2000]. The conversion of methanol to formaldehyde is the rate-limiting step in utilizing methanol and is regulated by increasing the amount of AOX enzyme in the cells. This is demonstrated by the observation that when the methanol concentration is increased suddenly, the growth of the cells is inhibited [Couderc R and Baratti J. 1980]. The formaldehyde produced in this reaction is then further oxidized either through a cytosolic dissimilatory pathway, which yields energy, or an assimilatory pathway for the generation of biomass [Gellissen G, *et al.,* 1992]. The first step in the assimilatory pathway is the activation of DHAS, which is present in the cytoplasm and is under the control of a methanol-induced promoter [Gellissen G. 2000; Tschopp JF, *et al*., 1987] The formaldehyde is converted into dihydroxyacetone and glucose 3-phosphate [Gellissen G. 2000]. In the dissimilatory pathway, the formaldehyde is converted to formate by FLD and then to CO_2 by FMDH [Gellissen G. 2000]. Peroxisome proteins such as AOX are thought to undergo rapid turnover [Tschopp JF, *et al*., 1987]. This turnover, combined with the enzyme's low affinity for O_2, means that the AOX protein levels can be high, e.g. up to 30% total cellular protein (TCP), and the peroxisomes can occupy up to 80% of the cell volume when *P. pastoris* is grown on methanol as a sole carbon source [Gellissen G. 2000]. As FMDH and DHAS are required for methanol utilization their levels can comprise up to 20% TCP [Gellissen G. 2000; Cereghino JL and Cregg JM. 2000].

1.3.1.4.2.5 Expression vectors

There are many commercially available vectors that can be used to express foreign proteins in *P. pastoris.*

Since some vectors do not contain a vector-copy of a secretion signal upstream from the multiple cloning sites, these vectors can be used for either intracellular expression or for extracellular expression by cloning the protein together with its own native secretion signal.

Other vectors contain a vector-copy of a signal sequence such as the alpha-mating factor pre-pro leader sequence (α-MF) prior to the cloning site, resulting in secretion of the desired gene product.

The first generation of *P. pastoris* expression vectors, such as pHIL-D2 or pPIC9 (Fig. 1.17), contained the functional histidine dehydrogenase gene (HIS4), which can be used as a selectable marker following transformation into his4 (histidine dehydrogenase-deficient strain) *P. pastoris* by the integration/transformation method of choice. In order to obtain high protein expression levels with these His^+ transformants, further analysis to detect tandem multi-copy integration events of the expression cassette is required.

Subsequently, a gene conferring resistance to GS418 (kanamycin) was added to these vectors to produce the pPIC9K expression vector (Fig. 1.17).

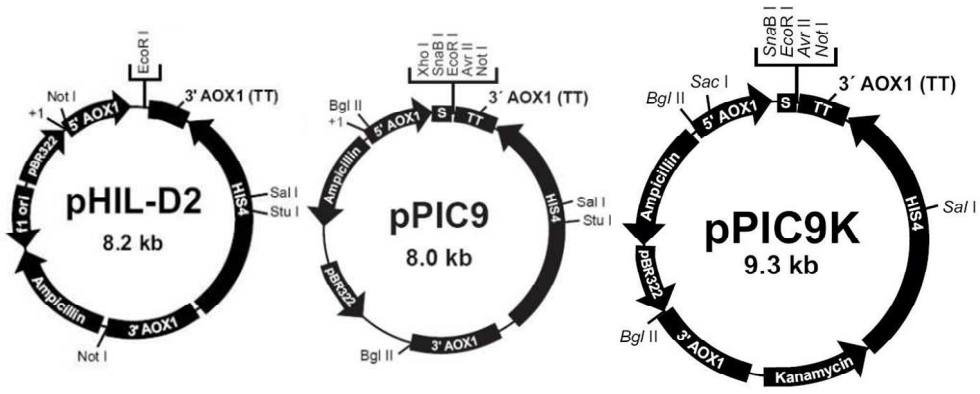


Fig 1.17: Vector diagrams of pHIL-D2, pPIC9 and pPIC9K.

This approach allows transformation and selection for His^+ transformants that are resistant to high levels of GS418 (kanamycin) and therefore can contain multiple copies of the expression vector. Several problems have, however, been documented with the use of the above-mentioned vectors. Firstly, the large size of these vectors (9.0–9.3 kb) makes in vitro cloning steps more difficult [Sears IB, *et*

al., 1998], and integration of such large vectors typically results in transformants that are genetically less stable [Romanos MA *et al.*, 1992]. Secondly, as not all applications require specific targeting for gene replacement, the 3'AOX1 gene region in the vector is not required. As the use of the HIS4 gene does not directly select for multi-copy integrants (and the gene itself is large), these constraints have led to efforts to find alternate selection markers in order to directly obtain multi-copy integrants, as well as to decrease vector size, resulting in the generation of vectors based on the ability of the *Sh ble* gene product to confer resistance to the antibiotic zeocin (Fig. 1.18). These zeocin-resistant vectors contain only the 5'AOX1 promoter region and the AOX1 transcription terminator as well as the *Sh ble* gene. Although these vectors cannot be used directly for AOX1 gene replacement, transformants can be selected for hyper-drug resistance containing multiple copies of the expression cassette. The Mut^{s} phenotype (aox1) may still result if the AOX1 gene is disrupted [Vassileva A, *et al.*, 2001]. In these zeocin-based vectors (3.3–3.6 kb), the expression cassette is also flanked by unique BamH1 and BglII sites that enable construction of a vector containing multiple, tandem expression cassettes (in vitro multimers).

Zeocin-resistant vectors are also available with α-MF (for secreted expression), no signal sequence (for intracellular or native signal extracellular expression) as well as with the choice of either the AOX1 or GAP promoter. This type of multimer cassette vector assembly has also been applied successfully for the co-expression of proteins in *P. pastoris*.

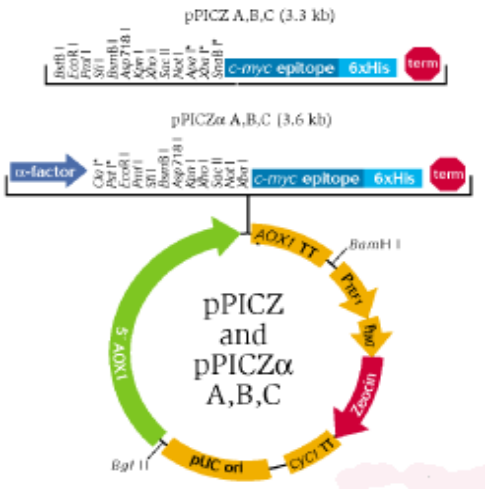


Fig1.18: Vector diagrams of pPICZ A, B, C and pPICZalpha A, B, C.

1.3.1.4.2.6 Expression plasmid transformation/integration into the yeast genome

P. pastoris is transformed by integration of the expression cassette into the chromosome at a specific locus to generate genetically stable transformants

[Sreekrishna K, *et al.,* 1997; Cregg JM and Higgins DR., 1995]. Chromosomal integration is more desirable than the use of episomal plasmid expression systems as episomal plasmids tend to have low copy number, and this will affect the amount of product expressed. The size of the plasmid may also affect the stability in the host since large episomal plasmids can be lost during repeated generations as they are mitotically unstable [Romanos MA *et al.,* 1992; Thiry M, and Cingolani D. 2002]. In addition, transformants containing episomal plasmids need to be cultured under continual 'selection based' media conditions in order to maintain the transformed population of cells [Romanos MA *et al.*, 1992]. This procedure may require the use of additives such as antibiotics, which in turn result in increased production costs [Thiry M, and Cingolani D. 2002].

Development of genetically stable expression strains is therefore highly desirable [Thiry M, and Cingolani D. 2002] with a rate of vector loss less than 1% per generation in the absence of selectable markers usually set as the target [Romanos MA. 1995]. Moreover, such integration vectors usually contain selectable markers that enable detection of the transformants.

Integration into the genome can occur via homologous recombination when the vector/expression cassette contains regions that are homologous to the *P. pastoris* genome and hence integration can occur via gene insertion or gene replacement (Fig. 1.19A–C). Integration by gene insertion can result in tandem multiple integration events due to repeated recombination events at a rate of 1–10% of transformants [Clare JJ, *et al.,* 1991]. Transformations that target gene replacement generally result in single copy transformants; however, gene replacement transformants are usually more genetically stable [Romanos MA *et al.,* 1992; Clare JJ, *et al.,* 1991]. Gene replacement is achieved by digesting the expression vector such that the 5' and 3' ends of the vector correspond to the 5' and 3' AOX1 regions of the AOX1 chromosomal locus. Transformation, therefore, results in site-specific eviction of the AOX1 gene (Fig. 1.19C). This event occurs at a frequency of 5–25% of the transformants [Sreekrishna K, *et al.*, 1997; Romanos MA. 1995]. The other transformants are either His^+ conversions or of the Mut^+ phenotype as a result of gene insertion events at either the his4 or AOX1 locus [Fig. 1.19A,B]. [Romanos MA. 1995].

The introduction of the expression cassette into the yeast chromosome can be achieved in a variety of ways including spheroplast formation, electroporation and lithium chloride treatments. The spheroplast transformation method has been used to generate multi-copy transformants by using vectors such as pPIC9K and pPIC9 [Greenwald J, *et al.,* 1998; Berger DH, *et al.,* 2002]. This method requires several steps with the risk that contamination of the yeast may occur. Also, over-digestion with the cell-lysing enzyme, zymolyase, can reduce cell viability. Electroporation has become increasingly popular and can be used successfully with zeocin-resistant vectors. This method requires fewer steps and the risk of contamination is reduced.

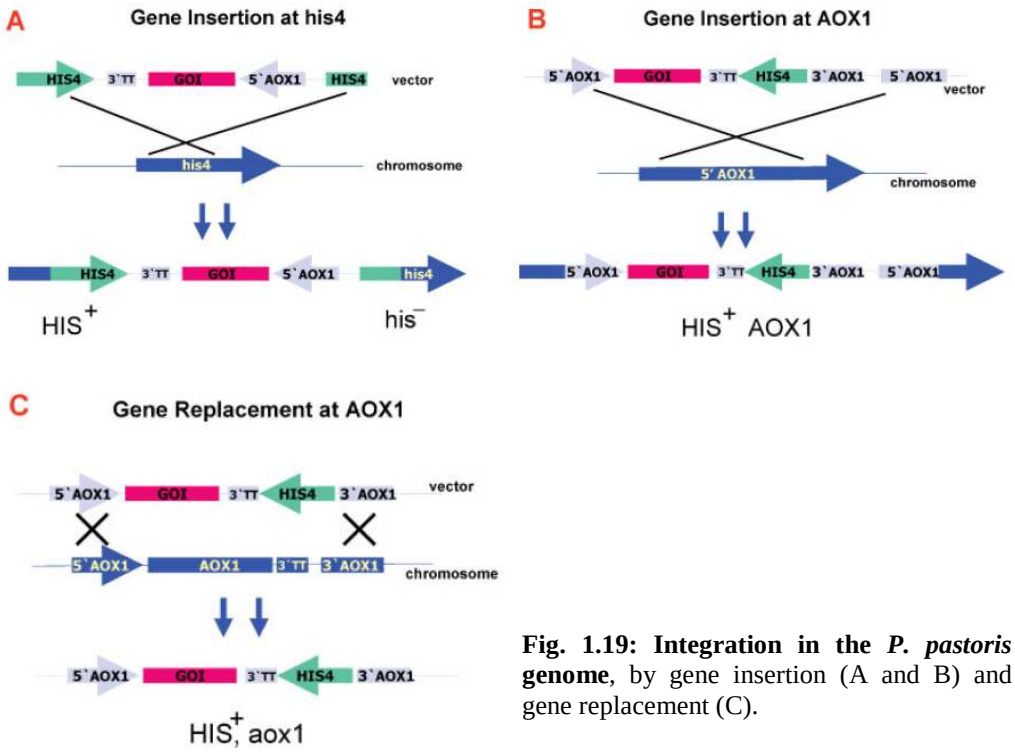


Fig. 1.19: Integration in the *P. pastoris* genome, by gene insertion (A and B) and gene replacement (C).

1.3.1.4.2.7 Choice of promoter

As mentioned previously, the AOX1 enzyme has a poor affinity for oxygen and therefore *P. pastoris* compensates for this property by up-regulating the AOX1 promoter to drive expression of the AOX1 gene and produce larger amounts of the AOX1 enzyme. In fact, the abundance of the AOX enzyme can reach 30% of TCP content when grown on methanol as a sole carbon source [Gellissen G. 2000; Cregg JM, *et al.,* 1993]. This strong AOX1 promoter can

therefore be used to drive the expression of recombinant proteins to high levels even with a single integrated copy of the expression cassette [Cregg JM, *et al.*, 1993; Clare JJ, *et al.*, 1991]. Other benefits of this promoter are that it can be switched off, as non-limiting amounts of carbon sources such as glycerol and glucose repress the AOX1 promoter at the transcriptional level and minimize the possibility of selecting non-expressing mutants/contaminants during biomass generation [Cregg JM, *et al.*, 1993; Inan M and Meagher MM. 2001; Cereghino GPL and Cregg JM. 1999; Katakura Y., *et al.*, 1998]. This characteristic also allows production of proteins that may be toxic to the *P. pastoris* cells, such as the anti-T cell Immunotoxin [Woo JH, *et al.*, 2002], by growing the cells initially in repressive media and then inducing the protein expression when the biomass has been well established [Waterham HR, *et al.*, 1997].

If the transformant used for expression has a Mut^+ phenotype, then this can result in the consumption of large amounts of methanol during the induction phase. Transformants with a Mut^s phenotype can therefore be used to reduce the amount of methanol required for expression.

Another promoter that has been used successfully with *P. pastoris* expression system is the constitutive YPT1 promoter, which is constructed from a GTPase gene product that is involved in the secretory pathway [Segev N, *et al.*, 1988]. Interestingly when the AOX1 promoter was induced with mannitol, significant amounts, 20.3 units/ml, of GUS were produced even though no methanol was present.

1.3.1.4.2.8 Rare codons and truncated transcripts

When expressing recombinant non-yeast proteins, the codons of the gene encoding the protein may not be optimal to accommodate high expression levels of protein. Codons that are common in some species may be rare in *P. pastoris* and so expression can become limited to the amounts of available aminoacyl-tRNA in the yeast cell [Romanos MA., *et al.*, 1992]. Premature termination of translation can occur when specific aminoacyl-tRNA are depleted [Lueking A., *et al.*, 2000; Clare JJ, 1991; Tull D, 2001; Eckart MR and Bussineau CM., 1996] and also transcription can be terminated if the DNA has a high proportion of AT bases [Sreekrishna K, *et al.*, 1997; Romanos MA., 1995]. Altering the codon bias as

well as increasing the proportion of GC bases in the DNA can be used to improve expression levels. An example of this approach can be found in the expression of mouse epidermal growth factor (mEGF) in *P. pastoris*, where the gene was made synthetically with a codon bias reflecting that of the yeast.53 The gene for α-amylase has also been synthetically made to improve codon usage and to also increase the percentage of GC bases [Tull D., *et al.,* 2001] in an analogous *P. pastoris* expression system. The β-glucanase gene has been similarly enriched with increased GC content [Olsen O, *et al.,* 1996], leading to higher expression levels of the corresponding protein, whilst an increase in GC content of tetanus toxin fragment gene also resulted in higher expression levels [Romanos MA *et al.,* 1992; Clare JJ, *et al.,* 1991]. Anti-human anti-T cell Immunotoxin has also been expressed in *P. pastoris* using the native cDNA sequence where it was found to result in a premature termination of the transcript due to AT-rich sequences. Optimization of the codons resulted in successful transcription and expression of the recombinant protein [Woo JH., *et al.,* 2002].

1.3.1.4.2.9 Multi-copy integration number

It is often desirable to select for transformants containing multiple integration events as such clones potentially express significantly higher levels of the recombinant protein. A further advantage of selecting for multi-copy transformants is that if there is a mutation in one particular copy of the expression cassette, arising from the integration process, then the protein that results from this mutant copy may not contribute as significantly to the total amount of protein expressed [Eckart MR and Bussineau CM., 1996]. Multiple integration events occur relatively infrequently at a rate of 1–10% [Chen YS., *et al.,* 2000]. The number of integrated copies of the expression cassette can affect the amount of protein expressed. As mentioned previously, a sensitive way to screen for multiple integration events is to use selective vectors that contain the *Sh ble* gene marker, which confers resistance to zeocin. Resistance to high concentrations of zeocin permits clones to be selected for multiple integration events as the *Sh ble* gene product binds to and inactivates zeocin in a dose-dependent manner [Monsalve RI, *et al.,* 1999; Vassileva A, *et al.,* 2001; Chen YS., *et al.,* 2000]. In a study reported by Vassileva et al. [Vassileva A, *et al.,* 2001], for example, the selection

effects for hyper-resistance to 100, 500, 1000 and 2000 mg/ml of zeocin were examined with the transformants analysed for copy number. It was found that transformants resistant to 100 mg/ml of zeocin generally contained one copy, those resistant to 500 mg/ml had two copies, those resistance to 1000 mg/ml had three copies and transformants resistant to 2000 mg/ml had four copies[Vassileva A, *et al.,* 2001]. In contrast, other studies by Sarramegna et al. [Sarramegna V., *et al.,* 2002] have found that transformants resistant to 1000 mg/ml zeocin had copy numbers of 15–25.

1.3.1.4.2.11 Intracellular expression

When expressing recombinant proteins in yeast, it is important to consider whether intracellular expression or extracellular secretion is the objective. The intracellular expression is therefore an alternative to secretion and usually does not result in glycosylation, thus in some cases providing a more desirable method. Purification of intracellular expressed proteins, however, can be more difficult than for secreted proteins as the target protein typically represents less than 1% of the total intracellular proteins [Rees GS, *et al.,* 1999]. However many proteins have been produced successfully in *P. pastoris* using intracellular expression systems, particularly membrane associated proteins [Vassileva A, *et al.,* 2001].

1.3.1.4.2.12 Folding pathway and the use of signal sequences

The folding of proteins usually begins with the formation of secondary structures [Hlodan R and Hartl UF., 1994] and the rapid generation of disulfide bonds in the endoplasmic reticulum (ER) [Holst B, *et al.,* 1996]. Many proteins, especially those that are naturally secreted, contain a proregion, which is required for correct folding and in some cases oligomerization. The mRNA of the pro-region can also assist in stabilizing the protein mRNA by the formation of local secondary structures and hence resisting degradation [Romanos MA *et al.,* 1992]. Although the pro-region is proteolytically removed to release the mature protein [Romanos MA., *et al.,* 1992; Holst B, *et al.,* 1996; Oka C, *et al.,* 1999]. The pro-protein is then transported into the Golgi where the pro-region is removed by dibasic endo-peptidases, such as kex2 or furin. From the Golgi, the recombinant

proteins are packaged into secretory vesicles and then delivered to the surface of the cell [Romanos MA., *et al.,* 1992].

In order to direct proteins into the secretory pathway, a specific signal sequence is required. The signal sequence is usually a short amino-terminal pre-sequence that typically contains a charged amino-terminal region followed by numerous hydrophobic residues (six to 15 amino acids) and a cleavage site recognised by signal peptidases [Romanos MA *et al.,* 1992; Paifer E., *et al., et al.,* 1994]. The use of a native signal sequence can also be used successful with *P. pastoris* secretory expression systems.

When deciding that protein secretion is the preferred approach to be used for a particular target protein, a choice of the specific secretion signal must be made. This selection can be based on the protein's own native secretion signal (if it has one), the *S. cerevisiae* alpha-mating factor pre–pro leader sequence (α-MF), the acid phosphatase signal sequence (PHO) or the invertase signal sequence (SUC2) [Li PZ., *et al.,* 2001].

The most commonly used signal sequence in *P. pastoris* secretion systems is the *S. cerevisiae* α-mating factor pre– pro leader sequence (α-MF). This sequence comprises a 19 amino acid signal peptide (pre-sequence), followed by a 60 amino acid pro-region. In generating the construct, it is usual for the expressed protein to be separated from the pre-region by an endopeptidase site. Upon translation, the signal sequence is removed by signal peptidase and pro-region cleavage site is recognized by the yeast kex2 protease (kex2p), resulting in the release of the mature, fully processed protein [Raemaekers RJM, *et al.,* 1999]. Kex2p is a type I membrane protein, located in the late Golgi compartments [Brake AJ, *et al.,* 1984; Henkel MK, *et al.,* 1999]. Kex2p is a serine protease [Smeekens SP., 1993; Henkel MK, *et al.,* 1999; Rockwell Nathan C, *et al.,* 1998], a member of the pro-hormone convertase family and is synthesized as a pre-proenzyme [Powner D and Davey J., 1998], which requires activation to the mature enzyme via autocatalysis [Davey J, *et al.,* 1998]. Kex2p recognizes basic pairs of amino acid residues such as Lys–Arg or Arg–Arg [Davey J, *et al.,* 1998; Smeekens SP. 1993; Henkel MK, *et al.,* 1999]. Further trimming of the amino-terminal Glu–Ala residue repeats also occur in the Golgi by the *ste13* gene product (ste13p). The ste13p is localized to the same late compartment as Kex2p

and is a type IV dipeptidyl aminopeptidase [Davey J, *et al.*, 1998; Li PZ, *et al.*, 2001]. Some of the expressed recombinant proteins that have been produced using this system are summarized in table 1.11.

Protein expressed	Function	Expression level
Bacteria		
Clostridium botulinum serotype F [BoNTF(Hc)] heavy chain fragment C	Antigen	205 mg/kg of cells
Escherichia coli AppA	Use in the animal feed industry to release inorganic phosphate	117 ± 1 U/mL
Escherichia coli phytase	Use in animal feed industry to improve phosphorus utilization	6.4 g/L
Gluconoacetobacer diazotrophicus exo-levanase	Fructose-releasing potential for high-fructose syrup production	0.46 g/L
Thermus aquaticus YT-1 aqualysin 1	Heat-stable subtilisin-type serine protease	1 g/L
Fungi		
Aspergillus oryzae tannase	Hydrolyses the ester and depside bonds of gallotannins and gallic acid esters	72 mg/L
Candida antartica CBM–CALB fusion protein	Lypase	1.3 g/L
Candida parapsilosis lipase/acyltransferase	Catalyses alcoholysis of esters	5.8 g/L
Rhizopus oryzae lipase	Catalyses breakdown of triglycerides	19 U/mL
Trametes versicolor cellobiose dehydrogenase (CDH)	Oxidation of reducing-end groups of cellobiose	225 U/mL
Trametes versicolor laccase (lcc1)	Phenoloxidase	23.9 U/mL
Trigonopsis variabilis D-amino acid oxidase (DAAO)	Flavoprotein, oxidoreductase	1.46 U/mg
Invertebrates		
Boophilus microplus BM95 antigen	Protects cattle from *B. microplus* infestation	550.0 mg/L
Pandalus borealis (shrimp) cathepsin L	Cysteine proteinase	60.0 mg/L
Vertebrates (non-human)		
Bothrops atrox moojeni venom batroxobin	Cleaves fibrinogen α-chain	7 mg/L
Bovine enterokinase light chain (EK_L)	Duodenal serine proteinase	350.0 mg/L
Equine lactoferrin (ELF)	Iron-binding protein, family of transferrins	40.0 mg/L
Hen egg H5-lysozyme	Bactericidal effects	2.1 mg/L
Hen egg white ovotransferrin	Iron-binding protein, family of transferrins	57.0 mg/L
Mouse endostatin	Tumour growth suppressor	133.33 mg/L
Ovine interferon-τ (r-oIFN-τ)	Antiviral and antiproliferative activity	391.7 mg/L
Porcine lactoferrin (PLF)	Iron-binding glycoprotein, family of transferrins	12.0 mg/L
Humans		
μ-Opioid receptor (HuMOR)	G-protein coupled receptor (GPCR)	4.0 pmol/mg
μ-Opioid receptor fused to green fluorescent protein	G-protein coupled receptor (GPCR)	1250 pmol/l
Angiostatin	Used in long-term therapy in suppression of metastases	108.0 mg/L
Anti-HBs Fab fragment	Prevention and treatment of hepatits B virus	50.0 mg/L
DNA topoisomerase I (hTopo I)	Role in DNA replication, transcription, recombination, chromosome assembly	11–58 mg/L
Dopamine D2S receptor	G-protein	3–13 pmol/mg
Granulocyte-macrophage colony-stimulating factor (hGM-CSF)	Regulation of activity of mature, differentiated granulocytes and macrophages	180.0 mg/L
Serine protease inhibitor SERpin C1-INhibitor (C1-Inh)	Plasma glycoprotein, inhibitor of inflammatory reactions	180.0 mg/L
Serum albumin	Binding and transport, colloid osmotic pressure	13.4 mg/g
Serum amyloid P (SAP)	G-protein, inhibition of DNA auto-antibody formation	12.0 mg/L
Transforming growth factor-β type II receptor extracellular domain (SolRII)	TGF-β antagonist, regulates cell proliferation and differentiation	20.0 mg/L
Plants		
Amaryllidaceae snowdrop agglutinin	Binding of α-D-mannose groups	80.0 mg/L
Arabidopsis thaliana core α1,3-fucosyltransferase	Biosynthesis of *N*- and *O*-linked oligosaccharides	1.0 U/L
Gsp (Panax ginseng C, medicinal peptide)	Potential use as drug against diabetes	800.0 mg/L
Maize cysteine proteinase (Mir1)	Seed germination, senescence	Insoluble/inactive
Rice α-amylase (Amy 1A/3D)	Hydrolysation of starch	340.0 mg/L
Rice β-D-fructofuranosidase (Osβfruct3)	Hydrolysation of sucrose	0.35 U/L
Solanum tuberosum (potato apyrase)	Phosphohydrolase, platelet-aggregation inhibition	1.0 mg/L
Thaumatococcus daniellii thaumatin II	Sweet-tasting protein	25 mg/L
Viruses		
Measles virus nucleoprotein (MeN)	Responsible for viral genome packaging	29% of total proteir

Table 1.11: Summary of a range of foreign proteins produced using the *Pichia* expression system since 2002. [S. M. Patrick, *et al*., 2005]

Although the α-MF pre–pro sequence has been used extensively, there are some instances where its application has been problematic. The main reason is due to the insertion of Glu–Ala dipeptide repeats between the amino-terminus of the mature recombinant protein and the Kex2p cleavage site. Although the Glu–Ala repeats do improve the proportion of correctly cleaved material by preventing steric hindrance of the kex2p cleavage site [Sreekrishna K, *et al.,* 1997], their presence results in the generation of a recombinant protein with Glu–Ala aminoterminal extensions. This can lead to N-terminal ragged ends due to differential amino-terminal processing because of the inability of the ste13p to process the large quantity of recombinant protein that is being produced [Brake AJ, *et al.,* 1984]. As the presence of these amino-terminal dipeptide extensions is undesirable for many recombinant protein applications, the Glu–Ala repeats between the mature protein and the kex2p can be omitted as part of the cloning approach in order to achieve a more authentic amino-terminus. Omission of these Glu–Ala repeats, however, can result in a decreased efficiency in the Kex2p cleavage specificity. In this case, secretion of the pro-protein cleavage products that have long amino-terminal extensions corresponding to 9–11 amino acids of the α-MF pro-region has been observed [Henkel MK, *et al.,* 1999]. The un-cleaved Glu–Ala repeats left as amino-terminal extensions can cause other problems in terms of the protein structure and function. [Koganesawa N, *et al.,* 2001]. These Glu–Ala repeats may also have an effect on the immunogenicity of the recombinant protein. [Monsalve RI, *et al.,* 1999].

1.3.1.4.2.14 Choice of culture condition for expression

The culture conditions used for *P. pastoris* expression systems are also an important factor to be considered in order to improve the productivity of a correctly processed protein.

Shaken-flask, small-scale expression methods are often the first stage employed in optimizing protein levels and selecting culture conditions. The levels of protein obtained from shaken-flasks are generally 10-fold lower than can be achieved with fermentors as the cell density is lower [White CE, *et al.,* 1994] and the extent of aeration is often more limited [Romanos MA., 1995]. Barr et al. [Barr KA, *et al.,* 1992] developed small-scale expression conditions that more

closely resembled the requirements of fermentor systems by ensuring high cell densities were generated through dramatic increases in the volume of the initial biomass generating cultures (1 l) and resuspending this biomass into small volumes of methanol induction media (50–75 ml).

The culture of *P. pastoris* transformants based on the AOX1 promoter can therefore be executed in two stages, firstly the generation of biomass in a repressive media, followed by the production phase where the cells are induced with methanol. The concentration of methanol used is also very important when optimizing expression levels. Increasing concentrations of methanol from 0.1 to 1.0% v/v was found to increase the expression levels of human β-2-glycoprotein I domain V for Mut^+ transformants [Katakura Y, *et al.,* 1998]. Typically methanol concentrations of 0.5–1.0% v/v are used in *P. pastoris* systems. The accumulation of methanol can, however, have negative effects on the cell growth [Romanos MA., 1995] and can lead to decreased protein expression levels [Sarramegna V., *et al.,* 2002]. Therefore, the optimal methanol level needs to be assessed for each specific protein produced. A methanol concentration of 3.1% v/v, for example, inhibited cell growth for production of β-2-glycoprotein I domain V [Katakura Y, *et al.,* 1998], whereas expression levels of procarboxypeptidase A2 increased 3-fold by increasing the methanol concentration in the induction media from 0.5 to 5% v/v in a Mut^+ phenotype transformant [Reverter D, *et al.,* 1998]. The components and pH of the media can also make a difference to expression levels. When the media is buffered to pH values between pH 3.0 and 6.0, the amount of proteolysis of the recombinant protein is often reduced [Sreekrishna K, *et al.,* 1997; Cregg JM, *et al.,* 1993].

The amount of proteolysis is also reduced if a peptone or yeast extract-based media is used. Another way to reduce proteolysis is via the addition of 1% casamino acids [Clare JJ, *et al.,* 1991]. Addition of L-arginine-hydrochloride or ammonium ions (in the form of ammonium phosphates) has also been used in an attempt to reduce proteolysis. Ammonium ions, in particular, seem to have the greatest effect at reducing proteolysis [Tsujikawa M, *et al.,* 1996].

The length of induction also has an effect on the extent of proteolysis. Proteolysis was found to increase over time when the number of viable cells decreased. This may be overcome to some extent by replacing the media with

fresh media to kick-start the production phase again and prevent accumulation of extracellular proteases.

Recombinant proteins expressed in *P. pastoris* systems have also found to be degraded rapidly if they contain PEST sequences. These sequences include the motifs XFXRQ or QRXFX, which are known to be degraded in the lysosome [Sreekrishna K, *et al.,* 1989]. Other additives such as the presence of 5mM EDTA can also assist in stabilizing the recombinant expressed protein [Sreekrishna K., *et al.,* 1997] and thus increase its expressed yield.

The temperature in the induction phase has also been found to affect not only the amount of proteolysis but also the amount of protein expressed. For example, the expression levels of α-galactosidase A produced in *P. pastoris* were increased when the temperature was reduced to 25°C [Chen YS, *et al.,* 2000]. The expression of human m-opioid receptor fusion protein was examined at numerous temperatures where it was found that the most functional protein was produced at 15–20°C, with 2- fold higher expression than obtained at 25°C and 4-fold higher than at 30°C [Sarramegna V, *et al.,* 2002]. Production of herring anti-freeze protein was also compared at 30 and at 23°C and again it was found that 10-fold more protein was produced at the lower temperature of 23°C. The amount of proteolysis occurring after 2 days of induction was also less at 23°C than at 30°C. These observations can be attributed to the 23°C cultures having a higher cell viability compared with the 30°C culture, even though the biomass in each culture was equal. The lower temperature can also be proposed to more efficiently stabilize the cell membranes and reduce the rate of protease release from the cells to the supernatant [Li ZJ, *et al.,* 2001]. The effects of decreased temperatures in the induction phase have also been reported in other studies [Brake AJ., *et al.,* 1984].

1.3.1.4.2.15 Glycosylation

Glycosylation is the most common post-translational modification to proteins preceeding protein secretion. Approximately 0.5–1.0% of the translated proteins in eukaryotic genomes are glycoproteins. Glycosylation occurs in the lumen of the endoplasmic reticulum after protein translation. (Fig. 1.20)

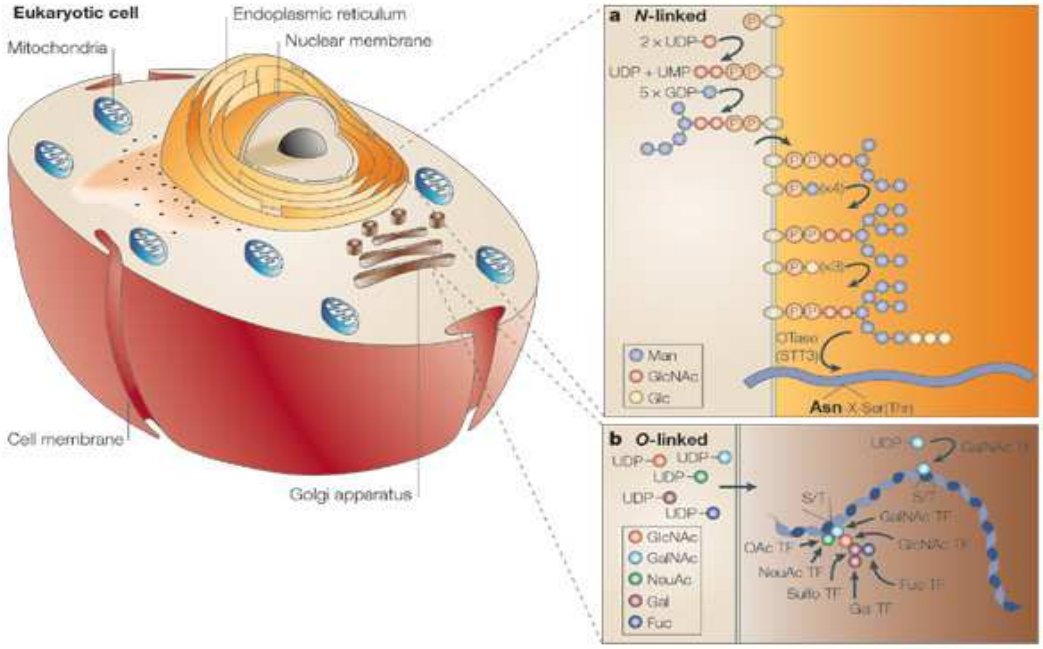


Fig. 1.20: Model for the biosynthesis of *N*- and *O*-linked glycoproteins in eukaryotes. [Modified from Christine M. Szymanski & Brendan W. Wren, 2005]

The initial stage in Asn-glycosylation is transfer of a pre-assembled $Glc_3Man_9GlcNAc_2$ unit from dolichyl pyrophosphate to amide groups of asparagine residues, in the lumen of the ER by the enzyme UDP-GlcNAc : dolichol PGlcNAc- transferase [Dennis JW., *et al.*, 1999; Lis H and Sharon N. 1993; Herscovics A and Orlean P., 1993]. This stage of glycosylation is similar in both yeast and mammalian cells. In the secretory pathway, the oligosaccharide is trimmed further by the removal of the three glucose residues by glucosidase II and I. The α-1,2-linked mannose residue is also removed by α-1,2-mannosidases to give $Man_8GlcNAc_2$ (Fig. 1.21).

The glycoprotein is then transported to the cis-Golgi where further processing occurs [Dennis JW., *et al.*, 1999]. In mammalian systems this additional processing includes further removal of mannose residues and incorporation of fucose (fuc), galactose (gal), N-acetyl-neuraminic acid (NeuAc), N-glycolylneuraminic acid (NeuGc), N-acetyl-galactosamine (Gal- NAc), N-

acetyl-glucosamine (GlcNAc) and sialic acid [Li PZ, *et al.,* 2001] by Golgi enzymes such as fucosyl-transferase and N-acetylglucosaminyl-transferase, galactosyl-transferase and sialyl-transferase [Khandekar SS, *et al.,* 2001]. Phosphate groups may also be incorporated into the oligosaccharide structure at this stage in the form of mannose-1-phosphate to create a phospho-diester linkages [Martinet W, *et al.,* 1998; Kobayashi H, *et al.,* 1986]. In yeast Golgi, however, mannoses are added and the oligomannose units can be linked α-1,6 to the α-1,3 mannose in the Manα-1,3-Manβ-1,4-GlcNAc$_2$ inner core [Herscovics A and Orlean P., 1993]. Glycosylation results in diverse structural heterogeneity of the protein population with some of this heterogeneity attributable to the tissue specific expression of Golgi glycosylation enzymes [Dennis JW., *et al.,* 1999]. Within a given cell, two different molecules of the same protein translate may have different oligosaccharide heterogeneity, even when they have been exposed to the same enzymes and glycosylation machinery. Some of this heterogeneity must therefore be target protein specific and due to features in the sequence recognized by the various glycosylation enzymes [Yan BX, *et al.,* 1999]. The chain length of the oligosaccharides can be dependent on the surrounding residues where hydrophilic residues may result in the sequon having longer length oligosaccharides [Lis H and Sharon N. 1993]. Furthermore, for the sequon Asn–Xaa–Ser/Thr, if Xaa is a proline, glycosylation is inhibited. A proline may also inhibit glycosylation if it is one amino acid displaced C-terminally to the sequon [Shelikoff M, *et al.,* 1996]. The Xaa residue is important, for example, if it is a hydrophobic residue, such as tryptophan [Yan BX, *et al.,* 1999] or phenylalanine [Shakin-Eshleman SH, *et al.,* 1996], then the initial step of addition of Glc$_3$Man$_9$GlcNAc$_2$ is inhibited. Negatively charged amino acids such as glutamic acid and aspartic acid also partially inhibit occupancy, whereas positively charged amino acids such as lysine, histidine and arginine promote core glycosylation and increase the occupancy levels [Yan BX, *et al.,* 1999].

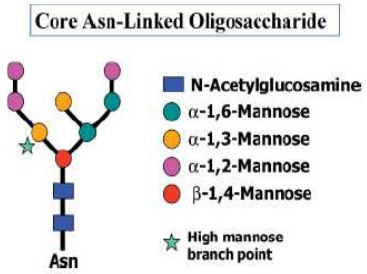


Fig. 1.21: Structure of the core Asn-linked oligosaccharide. The branch point where further oligosaccharides are added is indicated by the star.

The glycosylation recognition sequon in *P. pastoris*, as is the case in other eukaryotic systems, is Asn–Xaa–Ser/Thr [Yan BX, *et al.,* 1999]. The glycosylation is more readily achieved if the sequon is Asn–Xaa–Thr rather than Asn–Xaa–Ser. Bause and co-workers [Doyon Y, *et al.,* 2002] have shown that there is a 10- fold decrease in Km for the glycosyltransferase to hexapeptides with the sequence Asn–Xaa–Ser, which accounts for the reduced efficiency of glycosylation observed at this site [Doyon Y, *et al.,* 2002]. The finding that carboxypeptidase Y had 70% sequon glycosylation if the sequon was Asn– Xaa–Thr and only 25% glycosylated if the sequon is Asn–Xaa–Ser further validated this observation [Holst B, *et al.,* 1996].

The disposition of the sequon in the tertiary structure of the protein is important for glycosylation. There is a competition between folding and glycosylation in the ER. Upon translation the protein begins to fold and if the sequon is buried in the core of the protein, the glycosylation machinery may not have enough time to glycosylate the sequon before it is hidden [Shelikoff M., *et al.,* 1996]. The sequon itself may not be occupied, even when it is on the surface of the protein if the sequon residues are involved in strong hydrogen-bonding or have nearby disulphide bonds [Holst B, *et al.,* 1996; Simons JF, *et al.,* 1995; Doyon Y, *et al.,* 2002]. As glycosylation is an integral component of the secretory pathway, alterations to the protein that result in its slower exit from the ER will also promote more extensive glycosylation [Holst B, *et al.,* 1996]. When two tandem sequon sites occur in the primary sequence then hyper-glycosylation may occur whilst three tandem sites will result in complete hyper-glycosylation. It is further been suggested by various investigators that . . . Asn–Asn–Thr–Thr . . . is the sequon for hyper-glycosylation [Bretthauer RK and Castellino FJ., 1999]. The location of the sequon in relation to the C-terminus is often a determining factor for the extent of glycosylation. Sequons that are located close to the amino-terminus are exposed to the glycosylation machinery for longer than those that are close to the C-terminus [Tschopp JF, *et al.,* 1987; Shelikoff M., *et al.,* 1996]. The occupancy of some sequons may alter the rate of glycosylation of other sequons in the protein, while the presence of a surface accessible sequon does not guarantee occupancy.

1.3.1.4.2.15.1 Thermostability

The thermostability of some proteins has shown improvement upon glycosylation compared with the de-glycosylated counterpart [Tull D., *et al.*, 2001; Wang P, *et al.*, 2000;Yoshimasu MA, *et al.* 2002; Daly R. and Hearn MTW., 2004]. Glycosylation can also improve stability of proteins that are not normally glycosylated in their native state [Olsen O, and Thomsen KK. 1991; Minning S, *et al.*, 2001]. For this reason it is possible also to add glycosylation sites to improve the recombinant protein termostability [Jiang ST., *et al.*, 2002; Boraston AB., *et al.*, 2001]. The position of the oligosaccharide in the protein structure may therefore be more important in thermostability than the amount or length of the group [Olsen O. and Thomsen KK., 1991]. The increased thermostability observed for many proteins due to glycosylation can in many cases be attributed to the oligosaccharides contribution to the overall proteins structural stability [Olden K., *et al.*, 1985]. Also, glycosylation has been shown in many cases to help prevent proteolysis [Yan BX., *et al.*, 1999; Pratap J., *et al.*, 2000; Wang P., *et al.*, 2000]. The stability against proteolytic attack is probably due to the steric effects that the oligosaccharide poses to prevent the protease from the protein surface.

The post-translational steps involved in folding and glycosylation are therefore not equivalent in yeast compared with higher eukaryotes [Sadhukhan R., *et al.*, 1996].

1.3.1.4.2.15.2 O-linked glycosylation

O-linked glycosylation occurs both in mammals and yeast but may occur at different recognition sites [Jenkins N., *et al.*, 1996]. The oligosaccharides are added to the hydroxyl groups of serine or threonine residues in a protein [Li PZ., *et al.*, 2001; Bretthauer RK., *et al.*, 1999], and occur mainly in the Golgi apparatus [Dennis JW., *et al.*, 1999]. Mammalian O-linked glycosylation is composed of N-acetylgalactosamine, galactose and sialic acid [Cregg JM. and Higgins DR., 1995]. In yeast, such as *S. cerevisiae*, the O-linked oligosaccharides usually comprise one to five mannose residues linked α-1,2 in arrangement [Duman JG., *et al.*, 1998]. Although there is no definitive consensus sequon for O-linked glycosylation the probability of O-linked glycosylation is enhanced when there is

an unusual abundance of serine/threonine [Orlean P., *et al.*, 1991] or when there are proline residues near the serine/threonine [Herscovics A. and Orlean P., 1993]. O-linked glycosylation is observed particularly in glycoproteins that are involved in the yeast mating systems [Herscovics A. and Orlean P., 1993].

1.3.1.4.2.15.3 Immunogenicity

Although yeast expression systems such as *S. cerevisiae* and *P. pastoris* have the benefits of eukaryotic folding pathways, the glycosylation pattern is different to humans and other mammalian systems. This may or may not be a problem depending on the target protein and the applications in which it is used. For instance, certain carbohydrate groups are antigenic and Food and Drug Administration (FDA) now require full carbohydrate characterization to be conducted if it is has pharmaceutical applications [Jenkins N., *et al.*, 1996; Liu DTY., 1992] as glycosylation may change the function and characteristics of the recombinant protein [Eckart MR. and Bussineau CM., 1996]. Yeast expression systems, especially *S. cerevisiae*, hyperglycosylate with high mannose-type oligosaccharides and hence the recombinant protein can be recognized by mannose receptors when injected into mammalian species [Eckart MR. and Bussineau CM., 1996; Cregg JM., *et al.*, 1993; Cregg JM, Higgins DR. 1995]. *S. cerevisiae* glycosylation has terminal α-1,3 linked mannose residues and it is this residue that is thought to be antigenic. *P. pastoris* does not have this terminal link [Cregg JM., *et al.*, 1993; Scorer CA., *et al.*, 1993] as the *P. pastoris* α-1,3 mannosyltransferase activity is undetectable [Cregg JM, Higgins DR. 1995]; however, as it is still non-human glycosylation, it still may cause adverse immune responses [Eckart MR. and Bussineau CM., 1996; Cregg JM., *et al.*, 1993; Dennis JW., *et al.*, 1999; Lis H. and Sharon N., 1993; Goochee CF., *et al.*, 1991]. Glycosylation may therefore have effects on the protein clearance rate [Jenkins N., *et al.*, 1996; Tsujikawa M., *et al.*, 1996; Khandekar SS., *et al.*, 2001] .

1.3.1.4.2.15.4 Culture conditions to prevent glycosylation

To reduce the extent of glycosylation for a particular recombinant protein, the host cells (yeast or mammalian) can be cultured in the presence of tunicamycin [Boraston AB., *et al.*, 2001; Elbein AD., 1984]. Tunicamycin is a

sugar analogue that mimics the structure of UDP–GlcNAc and, as it is not incorporated into an oligosaccharide, tunicamycin is a competitive inhibitor of UDP–GlcNAc dolichol P-GlcNAc transferase [Villatte F., *et al.,* 2001]. Unglycosylated expression can also be achieved by expressing in the presence of N-benzoyl–Asn–Leu–Thr–N-methylamide where it competes with expressed protein sequons for Asn-linked glycosylation machinery [Huylebroek D., *et al.,* 1990]. Other alternatives to prevent or reduce the amount of glycosylation are to use mutant expression cells, which lack the genes that encode some of the glycosylation machinery.

Other alternatives that result in complete de-glycosylation is removal of the sequon by site directed mutagenesis (SDM) or by enzymatically or chemically de-glycosylating the protein post-expression. Enzymes, such as peptide-N-glycosidase F or endoglycosidase H, can accomplish enzymatic de-glycosylation. The reaction conditions may depend on the glycoprotein in question and choices of whether to denature the protein prior to de-glycosylation, which will require refolding, or whether to de-glycosylate the native protein. If the native protein is to be de-glycosylated considerations such as the reaction incubation time required to de-glycosylate the protein are important, as not all proteins are stable for long periods of time at the temperatures required by the de-glycosylation enzyme. If the protein is not stable, then compensations are required in the amount of enzyme needed in order to de-glycosylate the protein completely. Native protein de-glycosylation reactions are also less efficient than denatured proteins as the protein itself may sterically hinder the cleavage site so even stable proteins may require further optimization. SDM can be used to alter a sequon so that glycosylation is completely inhibited. If this is done, the protein functionality needs to be re-assessed to ensure that the mutation has not negatively affected activity and structure. SDM of the glycosylation sequon was assessed for CD154 protein where the Asn240 was mutated to Ala [Khandekar SS, *et al.,* 2001]. If SDM is chosen, then prior analysis of the sequon in relation to the tertiary structure needs to be determined because the position of the sequon may be such that it is not occupied anyway, as it could be buried in the core of the protein, or the Asn may be involved in H-bonding. If this is the case, SDM is not required and may result in disrupting the protein structure. Deglycosylation of proteins

may also be required in order to obtain their crystal structures. Glycoproteins are typically more difficult to crystallize; however complete de-glycosylation may not be possible due to its effects on stability and activity [Bretthauer RK. and Castellino FJ., 1999]. Alternatives such as treatment of the glycoprotein with Endo H (rather than PNGase F), which leaves the GlcNAc, may help to stabilize the protein [Curvers S, *et al.*, 2001].

1.3.1.4.2.16 Immunotoxins produced with *Pichia pastoris* expression system

In the table 1.12 the Immunotoxins produced in *Pichia pastoris* are summarized.

Immunotoxins	*Description*	*Reference*
ATF-SAP	Amino-terminal fragment of human urokinase (ATF) fused to the plant ribosome-inactivating protein saporin	Lombardi A., *et al.*, 2010
A-dmDT390-bisFv(UCHT1)	Bivalent anti-human T cell variable fragments fused to the truncated DT C-terminus	Woo JH, *et al.*, 2008
A-dmDT390-bisFv(G(4)S)	Bivalent anti-human T cell variable fragments fused to the truncated DT C-terminus	Woo JH, *et al.*, 2004 Liu YY, *et al.*, 2003 Woo JH, *et al.*, 2002
DT390biscFv(C207)	Diabody anti-monkey CD3 fusing to the truncated DT C-terminus	Kim GB, et al., 2007
scFvC6.5-syncyt2Aa1(mychis(6))	scFv anti p185(HER-2) cell surface glycoprotein fused to the toxin from the bacterium *Bacillus thuringensis*	Gurkan C and Ellar DJ, Aug. 2003 Gurkan C. and Ellar DJ, May. 2003

Table 1.12: Immunotoxins produce in *Pichia pastoris.*

Aim's work

E. coli is the host cell most frequently used for recombinant proteins production. However, this expression system has several disadvantages especially for the production of therapeutic proteins. For application purposes (e.g. therapeutic protocols) it is often needed to obtain high amounts of heterologous proteins, with rapid purification steps. The yeast *P. pastoris* allows the production of high amounts of recombinant proteins secreted in a protein-poor medium that makes the purification steps easy and quick. For these reasons I analysed the ability of *P. pastoris* to produce macromolecular drugs such as Immunotoxins. IL4(38-37)PE40 is a known and widely described Immunotoxin.

To produce this Immunotoxin in *P. pastoris* I followed these steps:

- production of IL4(38-37)PE40 in *E. coli*;
- production of IL4(38-37)PE40 in *P. pastoris*;
- IL4(38-37)PE40 gene optimization with the codon usage of *P. pastoris* (IL4(38-37)PE40-opti);
- comparison between IL4(38-37)PE40 and IL4(38-37)PE40-opti production;
- optimization of several induction conditions to improve the yield and to decrease the degradation level of recombinant protein;
- the produced proteins were preliminarily analyzed, with particular attention to the degradation phenomena taking place during culture and limiting the yield of the desired proteins.

2. MATERIALS AND METHODS

2.1 GENERAL MOLECULAR BIOLOGY AND BIOCHEMICAL TECNIQUES

2.1.1 PCR amplification of specific DNA fragments

The amplification of DNA fragments by polymerase-chain-reaction (PCR) was customized in order to fit the conditions required by specific templates and enzymes used in the reactions. All reactions were carried out using a GeneAmp PCR system 9700 thermocycler (Applied Biosystems).

2.1.1.1 Site-specific mutagenesis

To remove a restriction enzyme cleavage site or to change an amino acid codon the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) was utilized following the data sheet instructions.

2.1.2 Colony-PCR screening

For a rapid screening of *E. coli* colonies obtained after transformation with the ligation reaction, a PCR is performed directly on bacterial cells. Using a sterile tip, cells from a single colony are picked from the bacterial medium-agar plate and dissolved in 10 µl sterile, deionized water in a PCR-tube; the same tip is dipped in 50-100 µl of bacterial medium broth with appropriate antibiotics in a 96 wells plate, so that the positive bacterial clones can be recovered at the end of the screening. To each PCR tube are then added 15 µl of a concentrated reaction mix with: 2.5 µl of 10X reaction buffer (Fermentas), dNTPs mix (0.2 mM of each dNTP, Applied Biosystems), forward and reverse primers (10 pmoles each), 1 unit of Taq DNA polymerase (Fermentas). If necessary, also 5% DMSO is included.

Cycling programme:

94°C	5	min	
94°C	50	sec	35 cicli
55°C	50	sec	
72°C	1 min 30	sec	
72°C	7	min	
4°C	∞		

The PCR reactions are analysed by agarose-gel electrophoresis and staining with ethidium bromide. Amplificates of the expected size signify recombinant

clones, that can be recovered from the small LB cultures and propagated for extraction of plasmid DNA.

2.1.3 Plasmid DNA extraction from *E. coli* cultures

Single colonies picked from LB-agar plates (or from small LB cultures for colony-PCR screening) re-inoculated in 5 ml for small-scale preparations (miniprep), or in 50 ml for medium-scale preparations (midiprep) of LB broth additioned with the appropriate antibiotics (100 µg/ml Amp or 25 µg/ml Zeocin); the culture is grown O.N. at 37 °C with shaking (250 rpm) and cells are recovered by centrifugation (5 min, 10000 rpm at RT in minifuge for mini-preps; 10 min, 4000 xg at RT for midipreps) and the bacterial pellet is processed using the *Wizard Plus SV Minipreps DNA Purification System* (Promega) for mini-preps and the *PureLink HiPure Plasmid filter Purification kit* (Invitrogen) for midi-preps, according to instructions provided by the manufacturers. Purity and concentration of plasmid DNA are assessed by agarose gel electrophoresis and by spectrophotometric absorbance at 280 nm.

2.1.4 DNA digestion with restriction enzymes

Enzymatic digestion of plasmid DNA was performed according to the indications provided by manufacturers. Typically, in each reaction 5-10 units of enzyme are used to cut 1 µg of DNA. Reactions are usually incubated for 1 hour at the recommended temperature.

	Manufacturer	*Stock concentration*
XhoI	**New England Biolab**	**10 U/µl**
XbaI	**New England Biolab**	**10 U/µl**
PmeI	**New England Biolab**	**10 U/µl**
NcoI	**Roche**	**10 U/µl**
EcoRI	**New England Biolab**	**10 U/µl**
HindIII	**New England Biolab**	**10 U/µl**
SfiI	**Roche**	**10 U/µl**
NotI	**Roche**	**10 U/µl**

Table 2.1. List of restriction enzymes.

2.1.5 Ligation

Vectors and inserts for the preparation of all constructs were purified after agarose gel electrophoresis using the *QIAEX II Gel Extraction* kit (QIAGEN), and eventually ligated with T4 DNA ligase (Invitrogen). A 20 µl reaction is prepared in a clean eppendorf tube containing 100 ng of vector, a five or seven-fold molar excess of insert fragment, the provided ligation buffer and 2.5 units of enzyme. The ligation is incubated 2 hours at RT and ON at 4°C, after which 5 µl are used for the transformation of $CaCl_2$- competent *E. coli* cells (strain XL1blue).

2.1.6 DNA sequencing

The correctness of all plasmid constructs is confirmed by sequencing (BMR-Genomics, Padova, Italy). Analysis of the DNA sequences was performed using *Vector NTI Advance 10* software (Invitrogen).

2.1.7 Western blotting analysis

2.1.7.1 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic separation of proteins from crude extracts, supernatants or purified samples was performed according to standard laboratory procedures, using mini-gels with 5% acrylamide stacking and 10% separating slabs. Electrophoresis was conducted in Tris-Glycine buffer (25 mM Tris, 190 mM Glycine, 0,1% SDS), using the *Mini-Protean III Cell* apparatus (BioRad) under reducing conditions (samples were denatured by boiling for 5 minutes in Sample Loading buffer (4X) - 0.32 M TrisHCl pH 6.8, 6% SDS, 50% glycerol, 2% β-mercaptoethanol, 0.006 g Bromophenol blue-). A tension of 100 V was applied until the blue line (given by the bromophenol in the Sample Loading buffer) came out of the running gel. A protein size standard (*PageRuler Prestained Protein Ladder Plus*, Fermentas) was also loaded for molecular weight determination of the analyzed proteins.

2.1.8 Immunoblotting

2.1.8.1 Transfer of proteins on PVDF membrane

Proteins separated by SDS-PAGE were blotted on polyvinylidene fluoride (PVDF) membranes (*Immobilon–P*, Millipore) following the manufacturer's indications. The polyacrilamide gel and PVDF membrane were assembled as a

sandwich in a *Mini Trans-Blot Electrophoretic Transfer Cell* (BioRad) according to the manufacturer's instructions. For proteins to be transferred, a tension of 100 V was applied for 1 hour.

2.1.8.2 Immunodetection

After protein transfer, the PVDF membrane was incubated 30 min at 37 °C in blocking solution 5% w/v powder milk in 0.01% Tween-20, PBS under stirring. After blocking, the membrane was first incubated o.n. at 4°C with a primary antibody recognizing a specific epitope of the protein analysed. This was followed by incubation 45 min at r.t. with a horseradish peroxidase (HRP)-conjugated secondary antibody that interacts with the Fc portion of the primary antibody. Both antibodies were diluted in blocking solution and the membrane was rinsed twice for 5 minutes in 0.01 % Tween-20, PBS and twice for 5 minutes in PBS after each incubation. Bands corresponding to the immunoselected polypeptides were finally detected by a chemiluminescent reaction using the *ECL Western Blotting Substrate* (Pierce), according to the manufacturer's instructions, and visualized by development of a photographic plate (*Hyperfilm MP High performance autoradiography film* - Amersham Biosciences).

Antibody			**Dilution**	**Manufacturer**
Primary	Anti-PE	Rabbit polyclonal Ab	1:20000	SIGMA
	Anti-c-myc	Mouse mAb	1:2000	SIGMA
	Anti-His_6	Mouse mAb	1:1000	Ge-Healthcare
	Anti-IL4R	Mouse mAb	1:1000	Santa Cruz
	Anti-IL4	Mouse mAb	1:500	Santa Cruz
Secondary	Anti-mouse-HRP	Polyclonal Ab (HRP conj)	1:10000	DakoCytomation
	Anti-rabbit-HRP	Polyclonal Ab (HRP conj)	1:10000	SIGMA (A0545)

Table 2.3: Antibodies employed for the Western blotting analysis.

2.1.9 Protein quantification

2.1.9.1 Spectrophotometric quantification

Absorbance at 280 nm (Smart Spec 3000, Biorad) provides the quantification of purified proteins. One absorbance unit corresponds to 1.08 mg/ml of IL4PE40; it was determined using the software *Vector NTI Advance 10*.

2.1.9.2 Coomassie staining

A further means of protein quantification is provided by Coomassie staining after electrophoresis of purified proteins. SDS-PAGE gels are stirred for 20 min at r.t. in Coomassie solution, followed by decoloration in Destaining solution until bands are clearly visible. Amounts of proteins in discrete bands are assessed by comparison with known quantities of bovine serum albumin (BSA, Sigma).

2.1.9.3 Bicincininic Acid Assay – BCA - (SIGMA)

Proteins quantification through the BCA assay was performed according to the indications provided by the manufacturers.

2.2 RECOMBINANT IMMUNOTOXIN PRODUCTION AND PURIFICATION BY *E. coli* SYSTEM.

2.2.1 Bacterial strains and culture media

The *E. coli* Xl1blue strain (Stratagene) was used as the host for DNA manipulations and grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl; 2% agar in plates; 100 µg/ml ampicillin for the selection of plasmids). LB broth is used for transformation with Xl1-Blue cells. For growth, maintenance and protein expression with BL21(DE3)pLysS *E. coli* strains is used whether LB medium or 2XYT medium (1,6% tryptone, 1% yeast extract, 0,5% NaCl; 2% agar in plates; 100 µg/ml ampicillin for the selection of plasmids).

2.2.2 Bacterial expression plasmid

For the molecular cloning of DNA sequences the vector pET20b(+) (Novagen) was used.

pET20b(+) is an expression vector of 3716 bp carrying a multi-cloning site between T7 promoter and T7 terminator sequences.

Vector pET20b(+), by virtue of the pelB signal peptide, promotes the periplasmic sorting of the protein coded by the inserted sequence, also providing a poly-histidine carboxy-terminal tag. pET 20b(+) carries an ampicillin resistance and the pBR322 origin of replication

2.2.3 Cloning and construction of bacterial expression vectors

The cDNA encoding the IL4(38-37) permuted form was kindly provided by dr. Robert J. Kreitman, National Institutes of Health (Bethesda, MD) in the pRKL4389 plasmid. The PE40 sequence was already available in our laboratory.

The gene coding for the Immunotoxin IL4PE40 was insert in the pET20b plasmid, in frame with the *pelB* sequence at the 5', and the poly-histidine tag at the 3'.

2.2.4 Expression and purification of recombinant proteins

E. coli BL21(DE3)pLysS cells were transformed with the plasmid of interest through heat-shock, cultured in 1 liter of selected medium with 100 µg/ml of

ampicillin. The cells were grown up to A_{600}= 0.7, and induced with 1 mM IPTG for 3 h or o.n. The cells were harvested by centrifugation at 3000xg for 5 min. The cell pellet was kept at – 80°C before protein purification.

2.2.5 Cell growth conditions and optimization protein induction

To determine the optimal conditions that would result in a higher yield of the recombinant proteins, the inductions were performed under different conditions:

- medium of induction: LB medium or 2XYT medium
- time of induction: 3-4 h or o.n.

2.2.6 Subcellular localization of recombinant proteins by SDS-PAGE analysis

To determine the intracellular localization of recombinant proteins, the induced pellets were fractionated to extract the periplasmic fraction, the citoplasmic soluble fraction and the citoplasmic insoluble fraction.

The total pellet was kept as a control of total protein. Before harvesting the cells, 1 ml of culture was sampled and centrifuge at 10000×g for 1 min to remove the supernatant. Resuspend the pellet by mixing in 100 µl of PBS, giving a concentration factor of 10X. Add the 4X Sample Loading buffer for SDS-PAGE analysis.

When using vectors with *pelB* signal sequences, target proteins may be directed to the periplasmic space. The leader is necessary, but not sufficient for export into the periplasm. To extract the periplasmic fraction centrifuge 40 ml of the culture at 10,000×g for 10 min at 4°C. Resuspend the cell pellet in 30 ml of 30 mM Tris-HCl, 20% sucrose, pH 8. Then add 60 µl 0.5 M EDTA pH 8 (final concentration 1 mM), and incubate at room temperature for 10 min on the magnetic stir bar. Collect the cells by centrifugation at 4°C for 10 min at 10,000×g and remove all of the supernatant. Thoroughly resuspend the pellet in 30 ml of ice-cold 5 mM $MgSO_4$ and stir the cell suspension slowly for 10 min on ice. During this step, the periplasmic proteins were released in the solution buffer. Centrifuge at 4°C for 10 min at 10,000×g to pellet the shocked cells. Transfer the supernatant (periplasmic fraction) into a clean tube. Maintain the cell pellet on ice.

Concentrate 1 ml of the periplasmic fraction by TCA precipitation and resuspend the pellet with a concentration factor of 10X. Add the 4X Sample Loading buffer for SDS-PAGE analysis.

To isolate the soluble cytoplasmic fraction the mechanical disruption protocol was used. Through sonication the pellet was resuspend in 4 ml of cold 20 mM Tris-HCl, pH 7.5 to give a concentration factor of 10X. One µg/ml of lysozime was added and the mix was incubated 30 min at room temperature; the protease inhibitors (Complete – Roche- and 1mM PMSF) were added and the sample was sonicated (15 sec for 3 times). The lysate was centrifuged for 10 min at 14,000×g to separate the soluble (supernatant) from the insoluble (pellet) fractions.

The *insoluble cytoplasmic fraction* may consist of cell debris and aggregated protein known as inclusion bodies. The insoluble pellet was washed two times with 20 mM TrisHCl, pH 7.5. The solution was centrifuged at 10000xg per 5 min and the supernatant was removed. The pellet was resuspend in 1.5 ml 1% SDS with heating and vigorous mixing. One hundred µl of sample were removed and than combined with 4X Sample Loading buffer.

2.2.7 Inclusion bodies extraction through refolding and Immunotoxin purification by IMAC (Ni Sepharose 6 Fast Flow - GE Healthcare)

Inclusion bodies extraction and refolding - To purify the recombinant proteins from the inclusion bodies, the protocol of previous paragraph was followed for the isolation of the soluble cytoplasmic fraction. from 1 liter of *E. coli* culture

The pellet containing inclusion bodies was washed with 20 ml of 100 mM TrisHCl, 50 mM glycine. The pellet was weighed and resuspended with 100 mM TrisHCl, 50 mM glycine, 8 M urea to bring the inclusion bodies at the concentration of 20 mg/ml and incubated at r.t. for 60 min with stirring. The sample was centrifuged at 20000xg for 20 min. The supernatant was than transferred into a clean becker and 5 mM GSH and 0,5 mM GSSG were added. The mixture was incubate at 4°C o.n. with stirring.

The recombinant proteins were refolded through dialysis in buffers containing 0.1 M Tris, 0.4 M L-Arginine, 1 mM EDTA, 0.2 mM PMSF with decreasing concentration of urea: from 4M to 0M.

The last dialysis is carried out in the Ni-bind buffer utilized to load the refold proteins at the IMAC chromatography (50 mM Na_2HPO_4, 0.5 M NaCl).

IMAC protein purification – The "Ni Sepharose 6 Fast Flow resin" is highly stable and compatible with a wide range of common additives. This helps to maintain biological activity and increase product yield, while at the same time greatly expanding the range of suitable operating conditions.

The refolded sample was ultrafiltered (0.45 μm) and incubated 1 h at r.t. with the Ni-Sepharose 6 Fast Flow resin.

The Ni-bind buffer with 20 mM imidazole was used to wash the resin (10 ml at a time to reach an $OD_{280} \leq 0{,}05$). The tagged proteins were eluted with Ni-bind buffer with 500 mM imidazole; the elution was followed by spectrometric measurement at A_{280}. The fractions were analyzed by SDS-PAGE.

2.2.7 Immunotoxin purification by IMAC and refolding of inclusion bodies

IMAC protein purification - To purify the recombinant proteins was used an alternative protocol, where the proteins were purified and then refolded. Six molar Guanidine-HCl was used to solubilise the inclusion bodies. Since under denaturing conditions the His_6-tag is completely exposed, it could facilitate the binding to the Ni-resin.

One liter of induced culture was centrifuged at 10000xg for 5 min at 4°C. The pellet was resuspended in 100 ml of Lysis Buffer 1 (50 mM Na_2HPO_4, 0.5 M NaCl, pH 7.5) and 1 mM PMSF, 50 μg/ml DNasi and 10 mM $MgCl_2$ were added. The mixture was incubated 10 min at 4°C, then 1% Triton X-100 and 0.1 mg/ml of lisozyme were added. The sample was sonicated on ice 3 times for 20 sec and then centrifuged at 13000xg for 20 min at 4°C. The pellet was resuspended with 50 ml of Lysis Buffer 2 (50 mM Na_2HPO_4, 0.5 M NaCl, 6 M Guanidine-HCl) with PMSF, Triton X-100 and lisozyme. The sample was incubated 1 h at 30°C under agitation then centrifuged at 13000xg to 20 min at 4°C. The supernatant was collected and incubated with the Ni-resin for 1 h at r.t. with agitation. The resin was packaged in the column and washed with 10 ml of Wash buffer (Lysis buffer 2 with 20 mM imidazole, pH 7.5), to reach an $OD_{280} \leq 0{,}05$. The recombinant proteins were eluted with the Elution buffer (Lysis buffer 2 with 500 mM imidazole, pH 7.5); the elution was followed by by spectrometric

measurement at A_{280}. The fractions were analyzed by SDS-PAGE. Before loading the fractions in the poli-acrilamide gel, the samples containing Guanidine-HCl must be precipitated with 5% TCA to eliminated the denaturing agent.

Refolding - The fractions containing the recombinant protein were collected. The total sample was diluted 4-6 times with Lysis buffer 1 without Guanidine-HCl, to obtain a solution of 1M Guanidine-HCl. Add the Lysis buffer little by little, keeping the protein solution in the stirrer at 4°C. Centrifuge the sample to eliminate the incidental precipitate before dialysis start.

The recombinant proteins were refolded through dialysis in buffers containing 0,5 M NaCl, 50 mM TrisHCl, 0.4 M L-Arginine, 0.1 mM PMSF with decreasing concentration of Guanidine-HCl: from 0.5M to 0M.

The sample was concentrate with Amicon Ultra (cut-off 10 kDa - MILLIPORE) until to reach 0.5-1 mg/ml of protein concentration.

2.3 PRODUCTION OF RECOMBINANT IMMUNOTOXINS BY *P. pastoris* SYSTEM AND PURIFICATION

2.3.1 Yeast strains and culture media

The *E. coli* Xl1blue strain (Stratagene) was used as the host for DNA manipulations and was grown in low salt LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl; 2% agar in plates; 25 µg/ml zeocin for the selection of plasmids). The *P. pastoris* GS115 (his4) and SMD1168 (*his4*, *pep4*) strains (Invitrogen) were used for the recombinant proteins production.

P. pastoris was grown in YPD medium (1% yeast extract, 2% peptone and 2% dextrose; 2% agar in plates; 50 µg/ml zeocin for selection of transformants) and BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base without amino acids, $4*10^{-5}$% biotin, 1% glycerol) for growth. BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base without amino acids, $4*10^{-5}$% biotin, 0.5% methanol) was used as induction medium for production of recombinant proteins.

2.3.2 Yeast expression plasmids

pPICZα A, B, and C are 3.6 kb vectors were used to express and secrete recombinant proteins in *Pichia pastoris*. Recombinant proteins are expressed as fusions to an N-terminal peptide encoding the *Saccharomyces cerevisiae* α-factor secretion signal. The vector allows high-level, methanol inducible expression of the gene of interest in *P. pastoris.*

pPICZα contains the following elements:

- 5′ fragment containing the *AOX1* promoter for tightly regulated, methanol-induced expression of the gene of interest (Ellis *et al.*, 1985; Koutz *et al.*, 1989; Tschopp *et al.*, 1987a);
- α-factor secretion signal for directing secreted expression of the recombinant protein;
- Zeocin resistance gene for selection in both *E. coli* and *Pichia* (Baron *et al.*, 1992; Drocourt *et al.*, 1990);

- C-terminal peptide containing the *c-myc* epitope and a polyhistidine (6xHis) tag for detection and purification of a recombinant fusion protein (if desired);
- Three reading frames to facilitate in-frame cloning with the C-terminal peptide.

2.3.3 Cloning and construction of yeast expression vectors

The IL4PE40 gene used to produce the Immunotoxin in *E. coli*, was cloned in the pPICZα expression vector. The gene amplification was conducted with Pfu Ultra HF DNA Polymerase (2,5 U/µl – Stratagene). Amplified fragment was cloned in frame with the alpha-factor signal sequence. As described in the paragraph 1.3.1.4.2.12 of *Introduction*, to prevent the generation of a recombinant Immunotoxin with Glu-Ala aminoterminal extensions, the IL4PE40 gene was cloned usng the XhoI restriction site, and the Kex2 cleavage site was rebuilt through the forward PCR primer (Fig 2.1). For this reason the XhoI site contained in the IL4PE40 Immunotoxin sequence was removed by QuikChange® Site-Directed Mutagenesis Kit.

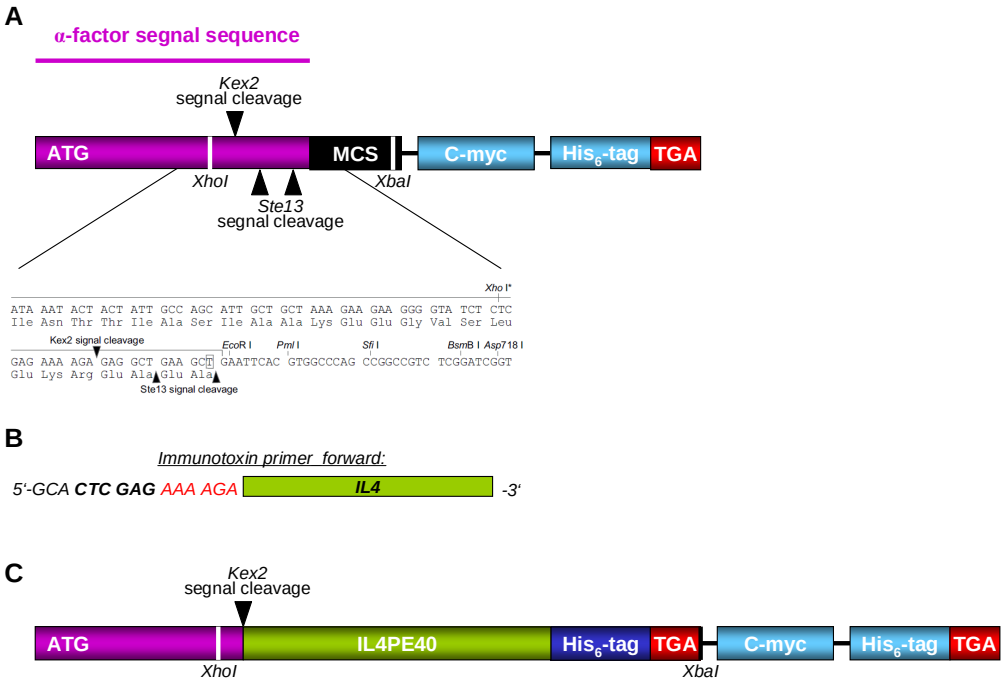


Fig. 2.1: A) Schematic diagram of pPICZα-cloning region. The *Kex2* and *Ste13* cleavage regions are evidenced. **B) Primer forward used to clone the Immunotoxins in the pPICZα-vector.** Highlighted: in black bold type the XhoI cleavage site, in red the codon coding from Lys-Arg (Kex2 cleavage site), in green the Immunotoxin start. **C) Schematic diagram of IL4PE40 cloning.**

2.3.3 Sequence optimization

To increase the yield of IL4PE40 Immunotoxin we have optimized the sequence with the codon usage of *P. pastoris.* A codon-modified cDNA of the Immunotoxin was generated by de novo gene synthesis by GenScript Corporation, Piscataway, NJ, USA.

2.3.4 *Pichia pastoris* transformation and screening of transformants expressing Immunotoxin

From five to 10 µg of plasmid containing the Immunotoxin gene were linearized with PmeI and then electroporated into GS115 and/or SMD1168 strains utilizing 400Ω, 1,5 V, and 25 µF with a Bio-Rad GenePulser (Bio-Rad Laboratories). Transformants expressing zeocin as selectable markers were obtained by spreading onto the YPD agar selection plate. The colonies obtained were re-streaked on fresh antibiotic selection plates to obtain pure colonies.

From 5 to 20 single colonies from each transformation experiment were grown in 5 ml of BMGY medium (1% yeast extract, 2% tripton, 100 mM potassium phosphate, pH 6.0, 1.34% Yeast nitrogen base, 4 x 10-5% biotin, 1% glycerol, 50 µg/ml zeocin) until culture reaches an OD_{600} = 2-6 (logaritmic phase growth). The cell pellet was resuspended in BMMY (1% yeast extract, 2% tripton, 100 mM potassium phosphate, pH 6.0, 1.34% Yeast nitrogen base, 4 x 10-5% biotin, 0.5% methanol, 50 µg/ml zeocin) at 1 OD_{600}. The methanol and the histidine were added every 24h. At the end of the induction period (48h or 72h) the culture medium were harvested by centrifugation and then subjected to Western blotting to check the Immunotoxin expression level.

2.3.5 Optimization of the induction conditions

To determine the optimal conditions that would result in a lower proteolysis and a higher yield of the IL4PE40 Immunotoxin, cultures were performed under different conditions:

- induction time (0h–72h)
- temperature of induction (20°C, 25°C, 30°C)
- effect of cell density at the induction start (1 OD_{600} or 10 OD_{600})
- presence of 1% glycerol during the induction;

- concentration of methanol added daily (0.25%, 0.5%, 1%);
- medium pH (pH 5, pH 6, pH 7);
- presence of 5 mM EDTA;
- condition culture before induction (inoculum induction in logarithmic phase or at saturation phase);
- Yeast strain (GS115 or SMD1168);
- Presence of an antifoam additive.

2.3.6 Expression in shake flask cultures

Induction with the culture in the log phase

For small scale induction a single colony was grown in 3-5 ml of BMGY medium o.n. to reach the log phase (2-6 OD_{600}). The culture was centrifuged at 1500xg for 5 min and the pellet was resuspended in 5 or 10 ml of BMMY medium. The small scale induction was performed to test the different optimized conditions.

For large scale production, a single colony was grown in 5 ml of BMGY medium for 6-8 hours. The whole culture was then transferred in 500 ml of BMGY medium to reach the log phase of growth. The culture was centrifuged at 1500xg for 5 min and the pellet was resuspended in BMMY medium (from 1 to 4 liter) at the optimized OD_{600}. The inductions were then conducted for 48h or 72h.

Induction with the culture at saturation

For small scale shake flask culture, a single colony was grown in 50 ml of YPD medium in a 250 ml flask for 2 days at 30 °C, resuspended in 30 ml of BMMY medium, and induced for 3 days with the addition of methanol (0,5%) every 24 h.

For large-scale culture, 50 ml of YPD medium was inoculated with 1 ml of frozen stock and grown for two days to establish a seed culture. Fifteen ml of seed culture were used to inoculate 300 ml of YPD medium and the inoculated medium was cultivated for 2 days. The cell pellet was resuspended in 200 ml of BMMY medium and induced for 3 days by adding methanol (0,5%) every 24 h.

In both cases, at the end of the induction period, the cells were removed by centrifugation for 15 min at 1600xg and 4°C and the supernatant was transferred

to new tubes and centrifuged again for 30 min at 3200xg and 4°C to remove cell debris. The protein containing supernatant was filtrated through a 0.45 µm syringe filter and stored at -20°C for further use. Five-hundred microliter of supernatant were precipitated with 10% TCA to analyze the protein production before proceeding to the purification step.

The collected supernatants were precipitated with 60% saturation with ammonium sulphate and resuspended in *binding buffer* (20 mM sodium phophate pH 7.4, 1.5 M NaCl, 10% glycerol, 20 mM imidazole, 1mM DTT), then dialyzed overnight against binding buffer to remove traces of ammonium sulphate.

2.3.7 Protein purification by IMAC (Ni Sepharose 6 Fast Flow - GE Healthcare)

The concentrated supernatant was centrifuged at 4°C, 20,000×g for 15 min and filtered through a 0.45 µm filter syringe and applied to the Ni Sepharose 6 Fast Flow resin. The sample was incubated for 1h at RT with the resin. This mix was then loaded onto the column and the flow-through was collected. The resin was washed with the binding buffer to reach an $OD_{280} \leq 0.05$ and the bound proteins were eluted with 500 mM imidazole dissolved in the binding buffer. The eluted proteins were detected by SDS-PAGE and Western blotting. The fractions containing the his_6-tagged protein were collected, concentrated by ammonium sulphate precipitation and dialyzed against PBS (137mM NaCl, 2,7mM KCl, 10mM sodium phosphate dibasic, 2 mM Potassium phosphate monobasic, pH 7.4). The final sample was sterilized with spin-x (0.2 µm) and quantified by SDS-PAGE and BCA protein Assay (Sigma).

2.3.8 Study of the glycosylation of non optimized IL4PE40 Immunotoxin producted in *P. pastoris*

The glycosylation sites were predicted by *CBS Prediction Servers* (www.cbs.dtu.dk/services/).

The presence of N-linked glycosylations on the IL4PE40 Immunotoxin produced in *P. pastoris* was initially analysed by in vitro deglycosylation of the supernatant using glycosidases EndoH and PNGaseF (New England-Biolabs) according to the supplier's instructions.

2.3.9 Native *Pseudomonas* exotoxin A (PE) cleavage test

The native PE was incubated for 1h at 30°C under agitation with: PBS as a control, only BMMY induction medium that was not inoculated with GS115, or BMMY cell-free after 48h of induction of GS115 transformed with the empty vector (αA) or BMMY cell-free with 1 mM PMSF (proteases inhibitor) after 48 h of induction of αA.

In a second cleavage test the native PE was incubated for 1 h at 30°C under agitation in αA BMMY with: 1 or 5 mM of EDTA or Complete EDTA-free1X (Roche).

The different samples were analysed by Western blotting.

2.4 CELL CULTURE TECHNIQUES

2.4.1 Cell lines and growth media

→ LNCaP: prostate carcinoma (left supraclavicular lymph node metastasis) cell line

→ PT45: pancretic carcinoma cell line

→ PACA44: pancretic carcinoma cell line

→ CF-PAC1: ductual adenocarcinoma (liver metastasis) from a patient with cystic fibrosis;

→ U251: glioblastoma cell line

All cell lines were grown in flasks at 37 °C, 5% CO_2, using the following medium: RPMI 1640 medium (with 40 mg/l folic acid, 2 g/l $NaHCO_3$) (Biochromag) supplemented with 10% Fetal Calf Serum (FCS), 2 mM L-Glutamine and antibiotics (100 U/ml penicilline and 100 µg/ml streptomycine-sulphate).

All supplements were added into the medium after sterilization through 0.22 µm filters.

The PT45, PACA 44, CF-PAC1 and U251 cell lines were grown in monolayer. To subculture the adherent cell lines the medium was removed, and the cells were detached with 0,25% (w/v) Tripsin-0.53 mM EDTA solution (SIGMA). An appropriate aliquot of the cell suspension was mixed with the complete growth medium.

The LNCaP and 293FT cell lines were cultured in monolayer on a film of Polilysine (SIGMA) and subcultured as described above for the other adherent cells.

Cell stocks were stored in liquid nitrogen tank at a concentration of 1 or $2*10^6$ cells/ml RPMI medium with 20% FCS and 10% DMSO.

2.4.2 Flow-cytometry analysis

2.4.2.1 Expression of IL4R by cell lines

PT45, CF-PAC1, PACA44, U251 and LNCaP cell lines were grown in T75 flasks to reach 50%-80% confluence and harvested by centrifugation at 1500 x *g*, 5 min , 4 °C. After counting with a hemacytometer, cells were resuspended in a blocking buffer (0.5% w/v BSA in PBS) to a concentration of $2*10^5$ cells/ml; 1

ml of the suspension was dispensed in each flow-cytometry tube and centrifuged as before. Cells were resuspended in 100 µl with 0.2 µg of anti-IL4R (Santa Cruz) and incubated on ice for 1 hour. Two washing steps followed, each with 2 ml of blocking buffer. For the detection of bound anti-IL4R cells were stained with an anti-mouse IgG-FITC secondary antibody (goat polyclonal from Beckman Coulter, 1:200 diluted). The incubation was for 30 minutes on ice in 100 µl of blocking buffer, followed by two washing steps with 2 ml blocking buffer each. Background fluorescence was assessed by staining the cells with anti-mouse IgG-FITC in the sample without the anti-IL4R. At the end of the staining, cells in each tube were resuspended in 0.5 ml blocking buffer and the mean fluorescence intensity (MFI) of each sample was determined using BD Facs Canto (BD Bioscience).

2.4.2.2 Binding of IL4PE40 to cells

The binding of IL4PE40 was analysed using PT45, U251 (IL4R+) and LNCaP (IL4R-) cell lines. The preparation of the samples was as above. In this case the cells were incubated with the increasing amounts of the IL4PE40 Immunotoxin and then with an anti-His_6 secondary antibody (mouse mAb from GE-Healthcare, 1:200 diluted in binding buffer) and then with the anti-mouse IgG-FITC tertiary antibody. At the end the cells were resuspended in 0.5 ml blocking buffer and the mean fluorescence intensity (MFI) was determined using BD Facs Canto.

2.4.3 Cell proliferation assay (XTT)

LNCaP, PT45 or U251 cells were dispensed in a 96-well plate (PT45 and U251: 5000 cells/well, LNCaP: 10000 cells/well) in 100 µl of RPMI medium. After 24 h different toxin or Immunotoxin dilutions were added to the cells and the culture was further incubated at 37°C for 72 h.

Proteins tested	PE40, and IL4PE40 from *E. coli*; PE (SIGMA)
Concentrations tested	From 10^{-6}M to 10^{-10}M

A mix with 1 volume of 1 mg/ml XTT and 3 volume of RPMI without phenol red was prepared. The prostate-specific antigen solution(PSA 1000X) was then added at the 1X final concentration. One hundred microliter of the mix were dispensed in each well. The absorption was measured at 450 nm after 20 min of incubation at 37°C.

Table 2.3: Description of PE40-based constructs

Construct	Description	Cloning plasmid	Cloning restriction sites
pET20bIL4PE40 XhoI ^{mut}	Removal of XhoI digestion site cointained within the Immunotoxin sequence	-	-
pPICZαIL4PE his	Immunotoxin with his_6-tag derived from bacterial expression vector	pPICZα	XhoI- XbaI
pPICZαIL4PE40 - opti	Immunotoxin with codon usage optimized for yeast	pPICZα	XhoI- XbaI
pPICZαIL4PE40 opti_mut1	Optimized Immunotoxin with 1 furin-like cleavage site mutated	-	-
pPICZαIL4PE40 opti_mut2	Optimized Immunotoxin with 2 furin-like cleavage sites mutated	-	-

3. RESULTS

3.1 Expression of IL4R in different cell lines

The expression of IL4R in several tumors was extensively described in literature [Puri, R. K., *et al.*, 1993; Puri, R. K., *et al.*, 1995; Puri, R. K., *et al.*, 1991; Obiri, N., *et al.*, 1993; Obiri, N., *et al.*, 1994; Hussain, S. R., *et al.*, 1994; Hoon, S. R., e*t al.*, 1997; Toi, M., *et al.*, 1992; Morisaki, T., *et al.*, 1992; Puri, R. K., 1996; Kreitman, R. J., *et al.*, 1994; Kawakami, K., *et al.*, 2000; Puri, R. K., *et al.*, 1996; Leland, P., *et al.*, 2000; Gallizi, J. P., *et al.*, 1989].

A commercial mAb anti-IL4R was used in different tumor cell lines: pancreatic tumor (LNCaP), prostate tumor (PT45, CF-PAC1 and PACA44) and gliobastoma (U251) to test the presence of IL4 receptor by flow-cytometry. The IL4R presence on U251 cell surface was previously verified by S.R. Husain [Husain S.R., *et al.*, 1998], for this reason this cell line was selected as a positive control. As shown in fig. 3.1 the pancreatic cell lines display a mean fluorescent intensity (MFI) higher than U251 and LNCaP cell lines. The latter shows a very low MFI. For this reason the LNCaP cell line was used as a negative control for binding and cytotoxicity assays.

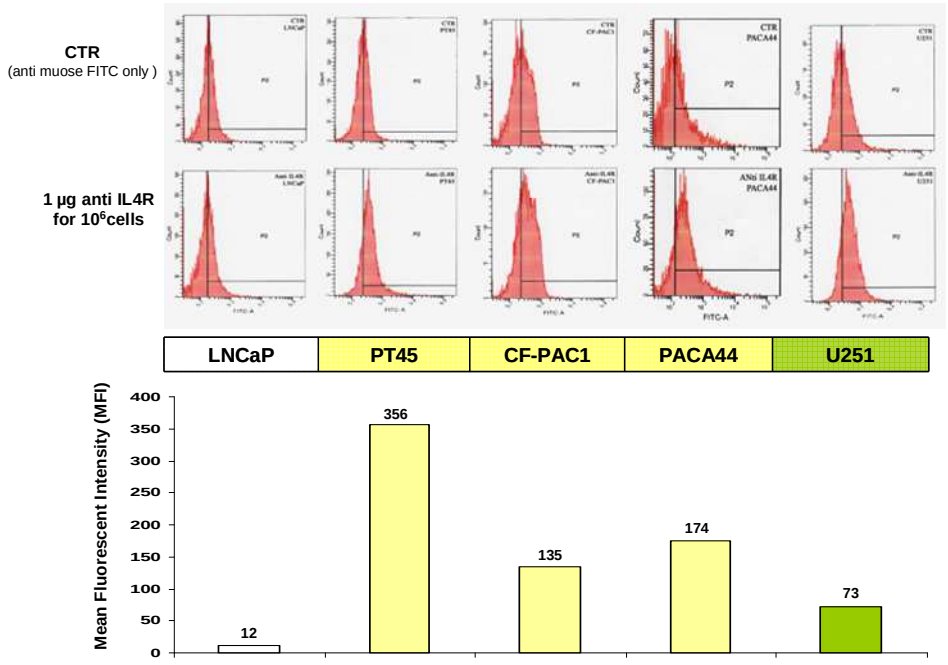


Fig. 3.1: Flow-cytometry in different tumor cell lines. The LNCaP (white), PT45, CF-PAC-1, PACA44 (yellow) and U251 (green) cell lines were analysed. For each cell line the upper box shows the fluorescence profiles obtained by staining 10^6 cells with 1µg of anti IL4R, followed by secondary anti-mouse IgG-FITC. In the graphic below the values of MFI are shown. $2*10^4$ events were recorded for each sample.

3.2 Cloning, expression and characterization of IL4(38-37)PE40 Immunotoxin obtained in the bacteria

3.2.1 Amplification and expression of IL4(38-37)PE40

The IL4(38-37) coding sequence was kindly provided by dr. Kreitman (National Institutes of Health. Bethesda, MD), whereas the PE40 sequence, a truncated version of Pseudomonas exotoxin A, derives from a construct already available in our laboratory.

The nucleotide sequence coding for PE40 was fused to the 3'-end of the IL4(38-37), generating a chimeric Immunotoxin in the pET20b(+) vector (Fig. 3.2 A). The C-terminal His_6-tag was exploited for purification and analytical purposes.

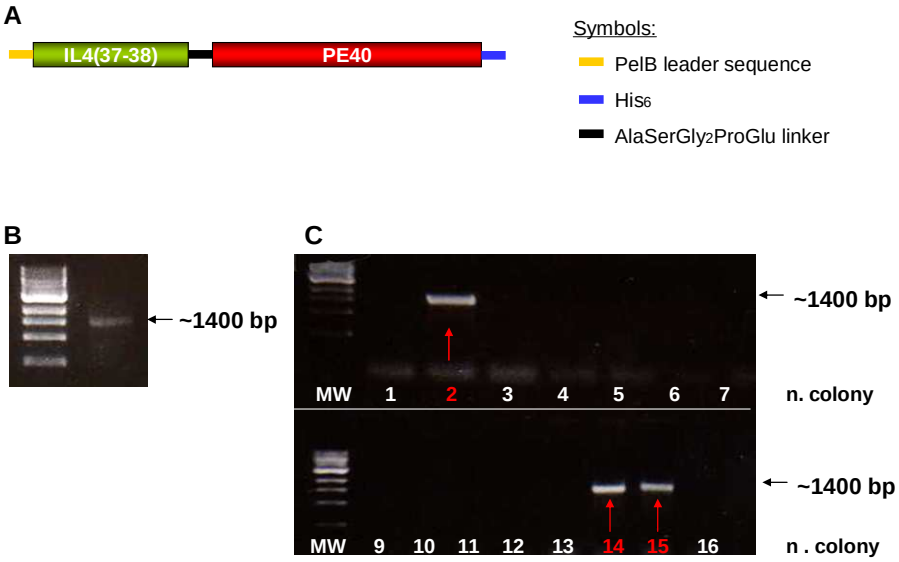


Fig. 3.2: A) The expression construct for IL4PE40 Immunotoxin; **B) IL4PE40 amplified through PCR; C) Screening of the colonies after transformation of *E. coli* cells with the ligation reaction:** The positive colonies were numbers 2, 14 and 15.

The small-scale expression of the IL4PE40 Immunotoxin in BL21(λDE3) pLysS *E. coli* yielded an induced protein of approximately 60 kDa, as visualized by Coomassie staining and immunoblotting with three different antibodies (Fig. 3.3), consistent with the expected size for a fusion between the IL4(38-37) (14 kDa) and PE40 (40 kDa).

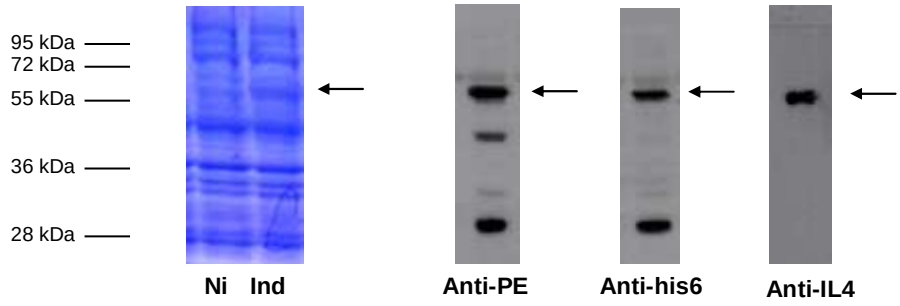


Figure 3.3 Immunotoxin expression in *E. coli*. First panel: Coomassie staining of an SDS-PAGE gel; Blotted membranes: probing with rabbit anti-PE serum (second panel), with mouse anti-his_6 antibody (third panel) and with mouse anti-IL4 antibody (last panel). Abbreviations: NI, bacterial pellet from 50 µl of non-induced culture; Ind, bacterial pellet from 50 µl of induced culture. Induction was carried out with 1 mM IPTG at 30 °C for 3 hours.

These preliminary induction data show that the Immunotoxin is expressed at low amount, suggesting the possibility of periplasmic accumulation. But from figure 3.4 it appears that most of the Immunotoxin is recovered in the inclusion bodies.

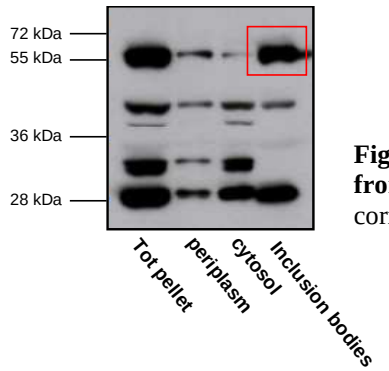


Fig. 3.4: Western blotting analysis of samples taken from different cellular districts. Each sample corresponds to 200 µl of culture.

To increase the yield of recombinant Immunotoxin, three induction variables were tested: the induction temperature, the time of induction and the culture medium. The best yield of recombinant protein was obtained after a 3h induction at 30°C, and the medium that allowed the highest Immunotoxin production was the LB medium (data not shown).

3.2.2 Immunotoxin extraction: refolding and purification under native conditions

A bigger culture was induced and processed for extraction, denaturation and refolding of inclusion bodies through the procedure described in Materials

and Methods (par. 2.2.4 of *Material and Methods*). The denaturing agent utilized was 8 M urea.

After the last dialysis of refolded IT in binding buffer, the sample was incubated 1h at r.t. with a resin that captures the his_6-tag (Ni Sepharose 6 Fast Flow – GE Healthcare; IMAC chromatography), and the recombinant Immunotoxin was eluted with 500 mM imidazole.

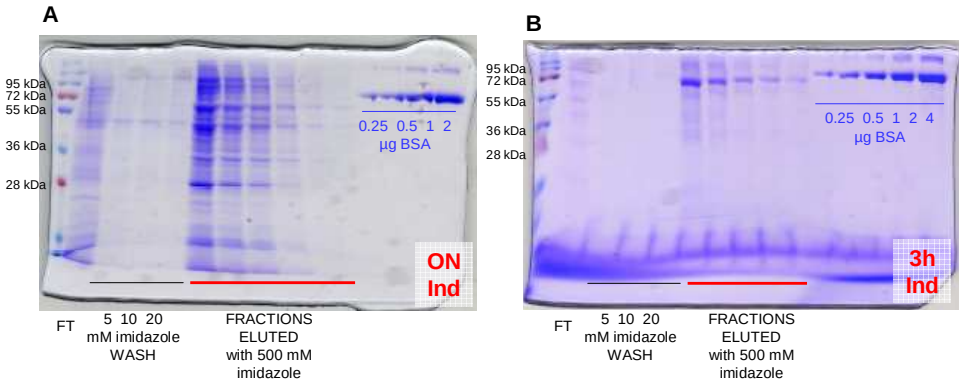


Fig. 3.5: Coomassie staining of SDS-PAGE of samples purified by IMAC under native conditions. **A)** Immunotoxin purification from a culture induced at 30°C o.n.; **B)** Immunotoxin purification from a culture induced at 30°C for 3h. Abbreviations: FT: flow-through collected after sample binding to the resin; BSA: bovine serum albumin; Ind: induction.

The purification was carried out on the refolded Immunotoxin derived from a culture induced o.n. and a culture induced for 3h (Fig. 3.5). The eluted fractions obtained from the o.n. culture were contaminated with other proteins or fragments of Immunotoxin (Fig. 3.5A). Moreover after 3h of induction the eluted fractions showed a single band with the expected molecular weight (Fig 3.5B). However, figure 3.6 shows that the amount of Immunotoxin binding to the resin is very low: the Immunotoxin is found in similar amounts in the refolded inclusion bodies (Fig. 3.6 red asterisk *) and in the flow-through (Fig. 3.6 black asterisk *).

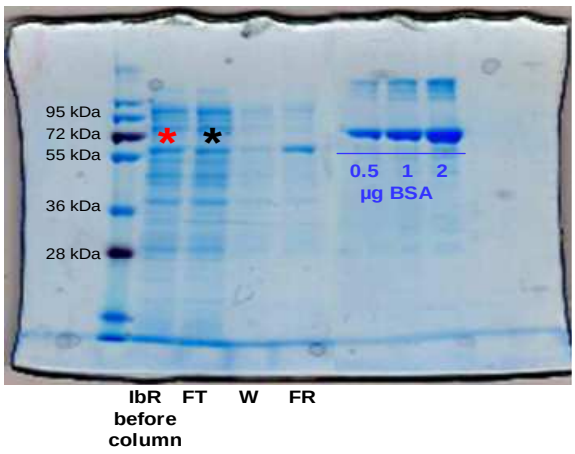


Fig 3.6: SDS-PAGE and Coomassie staining of samples from IMAC chromatography purification. Twenty microliter of IbR and FT were loaded on the SDS PAGE. The red asterisk and the black asterisk indicate the IL4PE in the IbR and in the FT respectively. Abbreviations: IbR: inclusion bodies after refolding; FT: flow-throught collected after sample binding to the resin; W: wash; FR: fractions collected; BSA: bovine serum albumin.

The purified Immunotoxin (after dialysis of the eluted protein in PBS and filter-sterilization) was concentrated and the final amount was about 200 µg recombinant protein from one liter of *E. coli* culture.

The low amount of Immunotoxin purified and the low binding to the resin suggest that under native conditions the His6-tag is masked, thus not allowing efficient retention by the resin. For this reason we next tried to purify the Immunotoxin following a protocol in which the recombinant protein was first purified and than refolded.

3.2.3 Immunotoxin extraction: purification under denaturing conditions and refolding

To prevent the loss of the IL4PE40 during the purification step, an alternative purification protocol was tested. The pellet of the induced culture was lysed, purified and then the eluted protein was refolded. For this protocol the denaturing agent used was 6M guanidine.

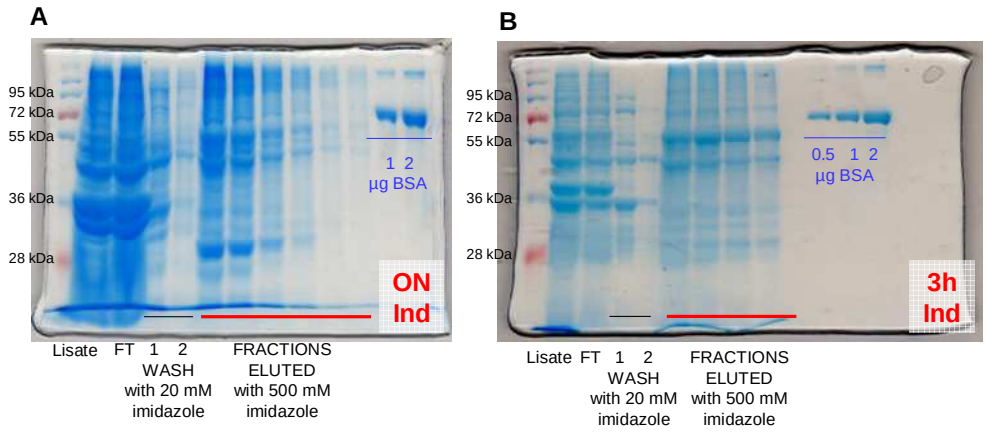


Fig. 3.7: Coomassie staining of two SDS-PAGE gels of samples after IMAC chromatography purification under denaturing conditions. **A)** Immunotoxin purification from a culture induced at 30°C o.n.; **B)** Immunotoxin purification of a culture induced at 30°C for 3h. Abbreviations: FT: flow-through collected after sample binding to the resin; BSA: bovine serum albumin; Ind: induction.

Figure 3.7 shows the IL4PE40 purification after o.n. induction or after 3h of induction at 30°C. As observed for the purification under native conditions, the Immunotoxin obtained after o.n. induction was less pure than that produced with a 3h induction, likely because the high culture density leads to the non specific accumulation of a high amount of bacterial proteins interacting with the resin. Adding 10% glycerol and 1 mM DTT in the lysis, wash and elution buffers, the

non-specific interactions between contaminant proteins and the resin decrease, and the purity degree of the Immunotoxin increases (Fig 3.8).

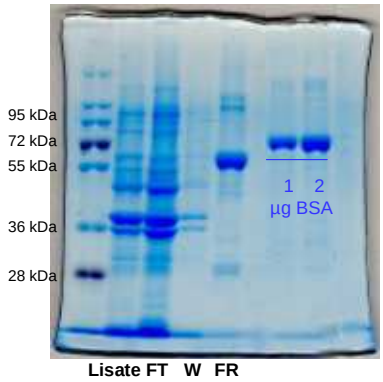


Fig. 3.8: Coomassie staining of the SDS-PAGE gel of samples from IMAC chromatography purification under denaturing conditions. Twenty microliters of lysate, FT, W and FR were loaded on the SDS PAGE. Abbreviations: FT: flow-through collected after sample binding to the resin; W: wash; FR: fractions collected; BSA: bovine serum albumin.

The best yield of purified Immunotoxin, after dialysis and concentration, was 600 µg from 1 litre of *E. coli* culture (data not shown).

3.3 Characterization of Immunotoxins produced by *E. coli*

3.3.1 Binding assay

To test if the Immunotoxin was correctly refolded in its active form, different amounts of recombinant proteins were incubated with 3 different cell lines: LNCaP (IL4R negative), U251 (IL4R positive) and PT45 (IL4R positive) (Fig. 3.9).

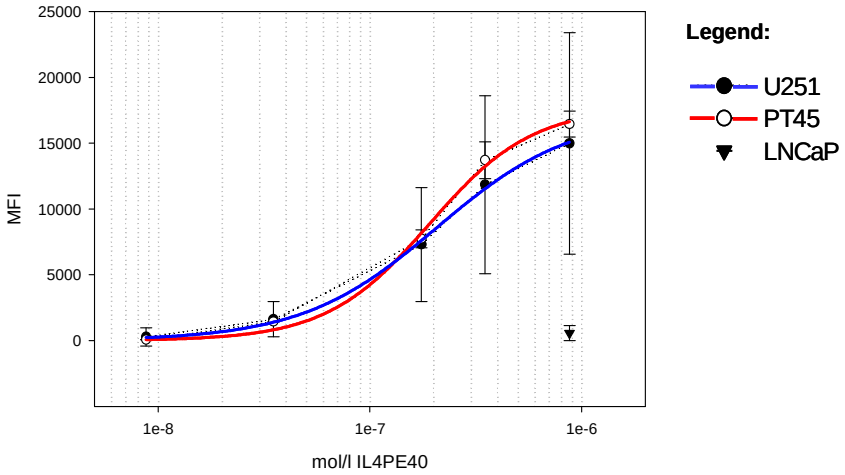


Fig. 3.9: Binding of the recombinant Immunotoxin on U251 (blue), PT45 (red) and LNCaP (black) cells. The curve was created by plotting MFI data from staining of U251 and PT45 cells (IL4R+) vs increasing amounts of Immunotoxin, and plotting MFI value from staining of LNCaP cells (IL4R-) at the highest tested concentration of Immunotoxin.

The binding assay showed that increasing the Immunotoxin concentration, the MFI value increases in the IL4R positive cell lines, whereas at the highest Immunotoxin concentration tested, the MFI value for LNCaP (IL4R-) was

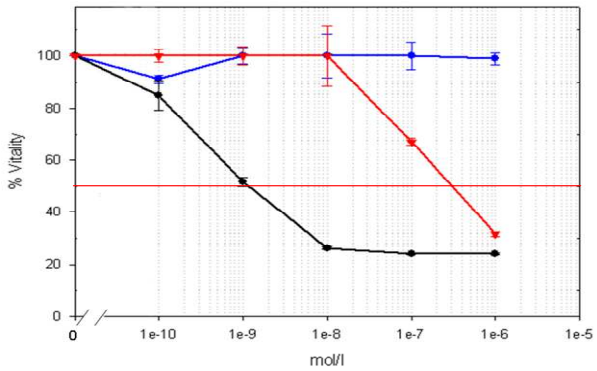
approximately zero. This means that the refolded Immunotoxin binds the cell lines that express IL4R, but not the IL4R negative cell line.

3.3.2 Cytotoxicity assay

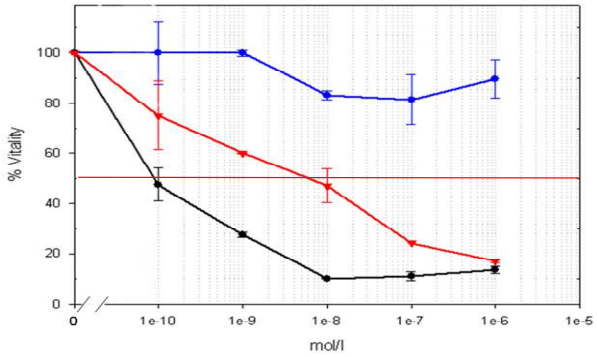
The cytotoxic potential was evaluated by testing on selected cell lines the effect of increasing concentrations of the Immunotoxin using the XTT assay. XTT is a reagent that develops an orange colour when it is metabolised by the mitochondria. The orange colour intensity is proportional to the amount of mitochondria and to the vitality of the cell culture.

For comparison, also wt PE, the native *Pseudomonas* exotoxin A, and the PE40 fragment (from bacterial culture) were tested. The curves in figure 3.10 clearly show that the Immunotoxin can inhibit the proliferation of cells expressing the IL4R. The concentrations of Immunotoxin that are capable to reduce by half the proliferation of the treated cells (IC_{50}) are reported in table 3.1. Remarkably, the Immunotoxin has a more potent action as compared to PE40 fragment which does not show cytotoxicity in the concentration range tested. This indicates that by adding the IL4 carrier molecule to the toxic domain, the new molecule is able to bind the receptor and to deliver the toxic domain, which in turn kills the cells.

(A) LNCaP



(B) U251



(C) PT45

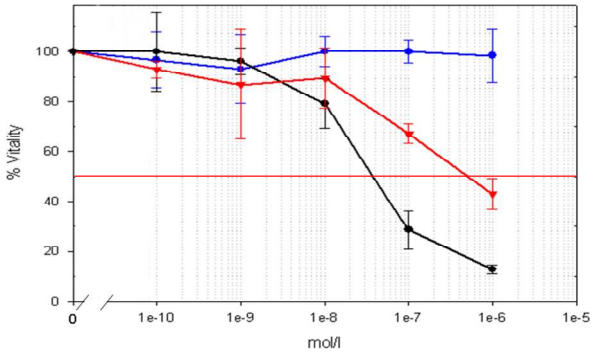


Fig 3.10: Cytotoxic assay. Effect of treatment with IL4PE40 Immunotoxin (red line), native PE (black line) or fragment PE40 (blue line) on the viability of LNCaP **(A)**, U251 **(B)**, PT45 **(C)** cells. The horizontal red line indicates the IC_{50}. Data are expressed as percentage of control sample (untreated cells).

	LNCaP	U251	PT45
		IC_{50}	
IL4PE40	$2*10^{-7}$	$6*10^{-9}$	$5*10^{-7}$
PE wt	10^{-9}	10^{-10}	$4*10^{-8}$
PE40	$>10^{-6}$	$>10^{-6}$	$>10^{-6}$

Table 3.1: Value of concentration inhibiting 50% of the maximal proliferation (IC_{50}) for IL4PE40 Immunotoxin, native PE (PE wt) and the fragment PE40 on IL4R+ lines U251 and PT45 and IL4R- cell line LNCaP.

The IC_{50} value of LNCaP (IL4R-) is comparable to that of PT45 (table 3.1). However, analyzing the native PE toxic activity in both cell lines, it is possible to observe that LNCaP cells are more sensitive to PE toxicity than PT45. This can explain the similar IC_{50}. In figure 3.11 the IC_{50} of IL4PE40 and native PE were compared: the specificity of the Immunotoxin is in inverse relation to the length of the red arrow and proportional to the specificity factor value.

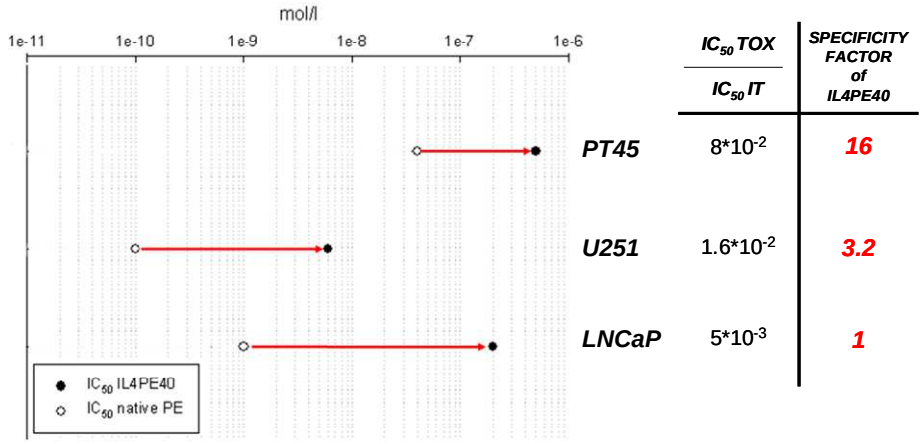


Fig. 3.11: Relationship between the toxin and Immunotoxin IC_{50} values in the different cell lines. The difference between the IL4PE40 IC_{50} and native PE IC_{50} on each cell line is shown with the red arrow. The specificity of the Immunotoxin is expressed as the normalization ratio between toxin or Immunotoxin IC_{50} values on positive cell lines (PT45 and U251) and IC_{50} on negative cell line (LNCaP). Abbreviations: TOX: toxin; IT Immunotoxin.

Binding and cytotoxicity data indicate that the IL4PE40 purified from *E. coli* is expressed in its active form.

3.4 Cloning of IL4(38-37)PE40 Immunotoxin in the yeast system.

3.4.1 Amplification of IL4(38-37)PE40 and cloning into pPICZalpha plasmid

The DNA sequence used to express the Immunotoxin in the bacterial system was utilised without modifications in the first pilot expression experiment in *P. pastoris* to verify the ability of these host cells to produce the Immunotoxin.

To clone the IL4PE40 gene into the pPICZalpha plasmid using the XhoI and XbaI restriction sites, a site specific mutagenesis was needed to remove the XhoI site within the Immunotoxin sequence. Six colonies were screened for restriction with 3 different enzymes: EcoRI recognizes 2 sequences into the bacterial expression vector, HindIII linearizes the plasmid and XhoI cuts once in the IL4PE40 wild type sequence but does not digest the mutagenized sequence. The EcoRI and HindIII restriction enzymes were used as a control.

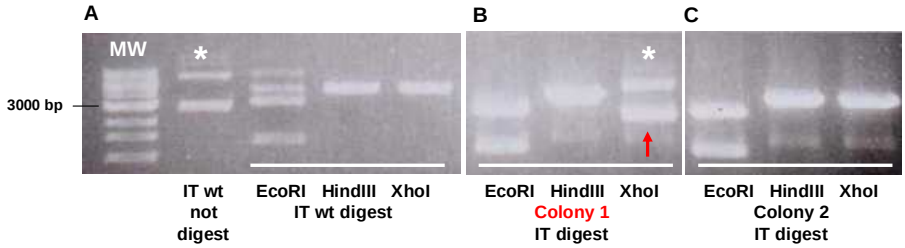


Fig 3.12: Screening of the colonies from mutagenesis reaction. A) In the first lane the MW standard was loaded. Sunsequent lanes: uncut IL4PE40, EcoRI digest, HindIII digest and XhoI digest, respectively; **B, C)** The DNA derived from colonies 1 and 2 was digested with EcoRI, HindIII and XhoI. The asterisks indicate uncut DNA. Abbreviation: MW: molecular weight; IT: IL4PE40 Immunotoxin.

As shown in figure 3.12, the non digested DNA appears with a typical pattern of the supercoiled DNA; the plasmid containing the IL4PE40 wild type sequence shows two bands at about 1300 bp and 3200 bp after cleaving with EcoRI whereas HindIII and XhoI linearize the vector (Fig. 3.12A). The figure 3.12 panel B shows that XhoI does not digest the colony 1 plasmid (second asterisk) whereas it linearizes the colony 2 DNA (fig. 3.12C). This means that in colony 1 the plasmid was mutagenized correctly. To confirm the mutagenesis the plasmidic DNA of colony 1 was sequenced by BMR Genomics.

The mutated IL4PE40 gene was amplified through PCR to insert the XhoI and XbaI restriction sites at the 5' and 3' respectively (fig 3.13B). The use of XhoI as restriction enzyme allows to produce the Immunotoxin with its native N-terminal (Par. 1.3.1.4.2.12 of *Introduction*).

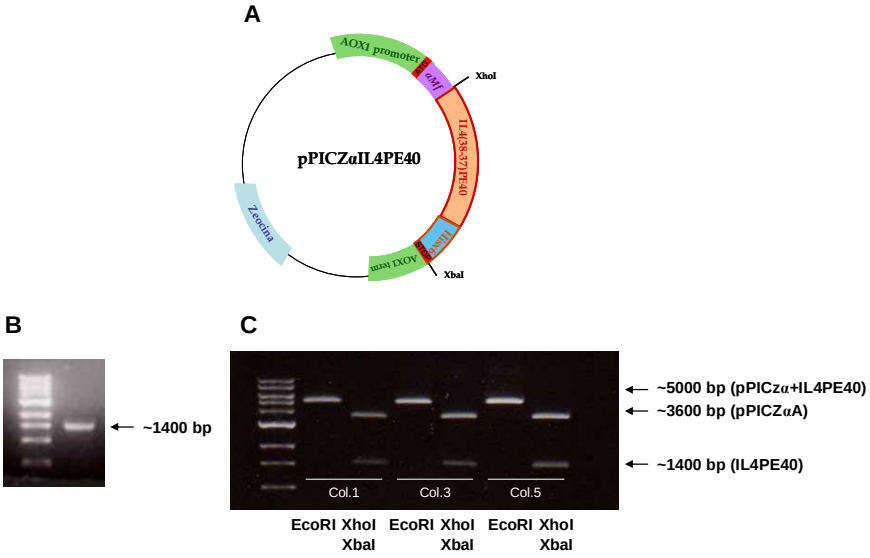


Fig. 3.13: **A) The pPICZα-IL4PE40 expression vector for *P. pastoris*; B) IL4PE40 amplified through PCR; C)** After screening of colonies derived from the ligation reaction, **three colonies were digest with EcoRI and XhoI and XbaI as a further control.**

The digested PCR fragment was inserted into the pPICZα plasmid. This expression plasmid contains the α-mating factor signal sequence (αMf) that drives the secretion of the recombinant proteins in the culture medium. The new gene was inserted at the 3' of αMf, without native ATG (Fig. 3.13A).

Twenty colonies derived from the ligation reaction were screened and the colonies 1, 3 and 5 were found positive. To confirm the presence of the Immunotoxin gene in the vector, the DNA plasmid of these colonies was digested with EcoRI that linearized the construct and with XhoI/XbaI that cut within the cloning restriction sites (Fig. 3.13C); moreover one construct was also sequenced also by BMR Genomics. For the electroporation of *P. pastoris* the colony 1 was chosen.

3.4.2 Screening of *P. pastoris* transformed with IL4PE40 construct

The yeast transformation protocols are described in the par. 2.3.4 of *Materials and Methods*.

Thirteen colonies were induced to identify the clone with the best yield (fig.3.14). The clone 5 was chosen for the medium and large scale inductions.

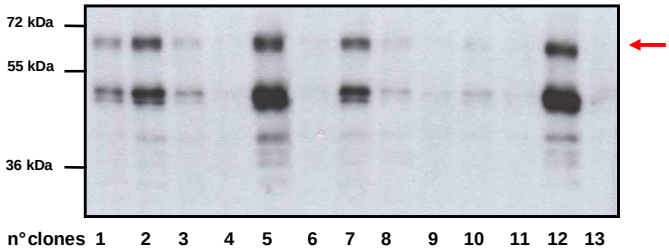


Fig. 3.14: Screening of 13 clones derived from transformation by electroporation.

3.4.3 Purification and quantification of IL4PE40 from 1L of culture

To quantify the Immunotoxin production, the IL4PE40 clone 5 was induced in 1 liter of BMMY medium. After 72h of induction, the culture was centrifuged and the supernatant was collected. To load the supernatant in the IMAC chromatography, sample concentration is needed. To identify the correct percentage of saturation of ammonium sulphate allowing the Immunotoxin precipitation, a fractionated precipitation was conducted (Fig. 3.15)

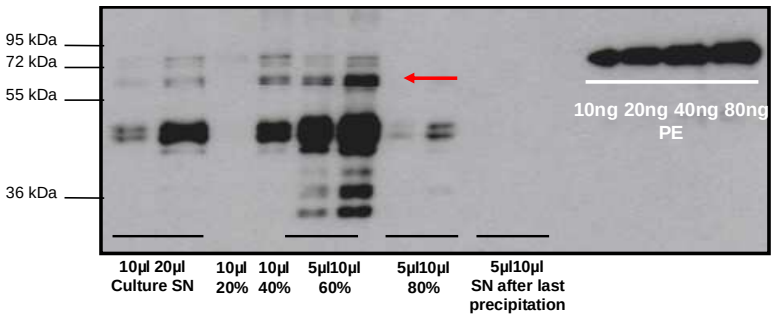


Fig. 3.15: Western blotting of ammonium suphate-precipited proteins. The WB was stained with the rabbit anti-PE as primary antibody and with the anti-Rabbit-HRP as secondary antibody. The different precipitated samples were loaded concentrated 10-fold as compared with the culture supernatant. Abbreviation: SN, culture supernatant; 20%: culture supernatant precipitated with 20% of saturation of ammonium sulphate; 40%: 20% supernatant precipitated with final 40% of saturation of ammonium sulphate; 60%: 40% supernatant precipitated with final 60% of saturation of ammonium sulphate; 80%: 60% supernatant precipitated with final 80% of saturation of ammonium sulphate; SN after last precipitation, 10X concentrated supernatant after centrifugation of 80% saturated of supernatant.

The Immunotoxin precipitates between the 40% and 60% of saturation of ammonium sulphate. Since in a Coomassie staining 20% of saturation does not result in proteins precipitation (data not shown), the supernatant of induced Immunotoxin inducted was precipitated directly with a 60% ammonium sulphate saturation.

After 1h incubation with ammonium sulphate at 4°C and centrifugation of the solution the proteins pelleted were resuspended in binding buffer, and dialyzed

o.n. against the same binding buffer to remove the ammonium sulphate traces. The supernatant equilibrated with binding buffer was incubated 1-2 h with the resin that binds the his_6-tag and, after washing the resin with 20 mM imidazole, the Immunotoxin was eluted with 500 mM imidazole. Since the SDS-PAGE stained with Coomassie does not show the band corresponding to the molecular weigh of the Immunotoxin (data not shown), the fractions were analysed by Western blotting (Fig 3.16).

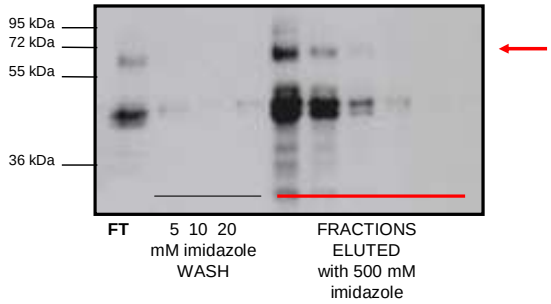


Fig. 3.16: Western blotting of samples from IMAC purification step of *P. pastoris* supernatant. Twenty microliters of each sample were loaded. Abbreviations: FT: flow-through collected after sample binding to the resin; BSA: bovine serum albumin; Ind: induction.

As shown in figure 3.16 traces of Immunotoxin are present in the flow-through, the amount of purified protein is very low and mostly degraded. In fact, after dialysis carried out to remove the imidazole and concentration, the final Immunotoxin yield was 1 µg from 1 liter of culture.

3.4.4 IL4PE40 optimization for the production in *P. pastoris*

3.4.4.1 IL4PE40 codon optimization for production in *P. pastoris*

To increase the recombinant protein amount produced by yeast, DNA sequence was optimized to the host strain codon usage.

The IL4PE40 sequence was codon optimized by GenScript Corporation (Piscataway, USA). The OptimumGene^{TM} algoritm used takes into consideration a variety of critical factors involved in different stages of protein expression, such as codon adaptability, mRNA structure and various cis-elements involved in transcription and translation.

Before IL4PE40 gene codon optimization, some features, such as the glycosylation sites and the restriction sites important for the subsequent cloning, were studied.

To identify the glycosylation sites the IL4PE40 sequence was inserted into the prediction servers named NetNGlyc and NetOGlyc (www.cbs.dtu.dk/services/) to search for N- or O- glycosylation sites respectively. The NetOGlyc server did not find any target sequence, however 2 putative N-glycosylation sites are present in the Immunotoxin sequence (Fig. 3.17A-B). To confirm the presence of glycosylation sites, 10 µl of supernatant of IL4PE40 culture were incubated with 1 µl of PNGaseF and 1 µl of EndoH, two N-glycosydases that remove the carbohydrate residues from proteins. As shown in the western blotting of figure 3.18C in the lines without the enzyme (-) the Immunotoxin shows 2 bands (corresponding to two different glycosylation conditions), moreover when the enzymes were added to the recombinant protein only 1 band is evident because the sugar chains were removed. To exclude a possible *in vivo* immune reaction against the sugar chains of the recombinant Immunotoxin, the N residues recognized by the software of prediction were changed in two Q residues that maintain the negative charge of amino group.

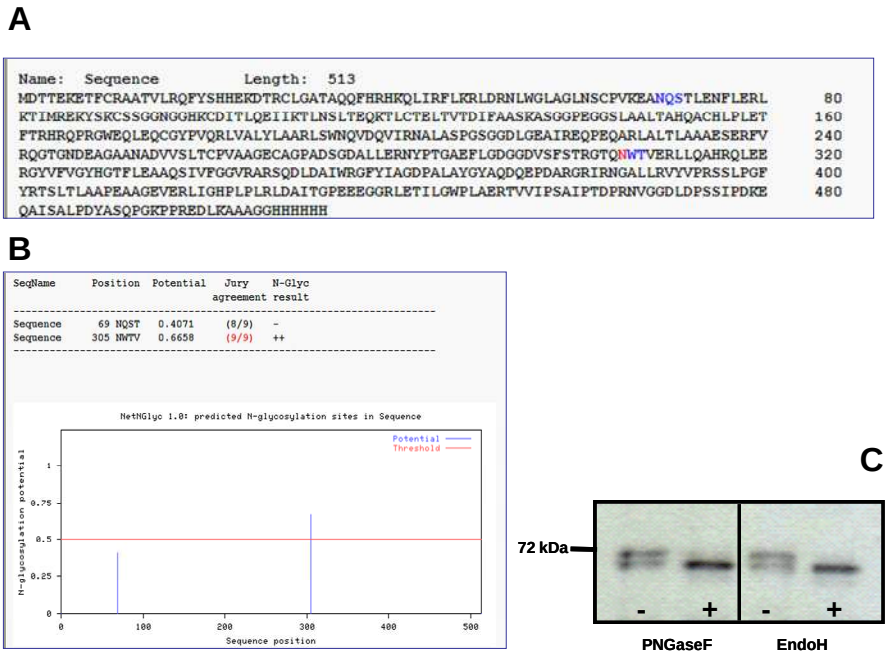


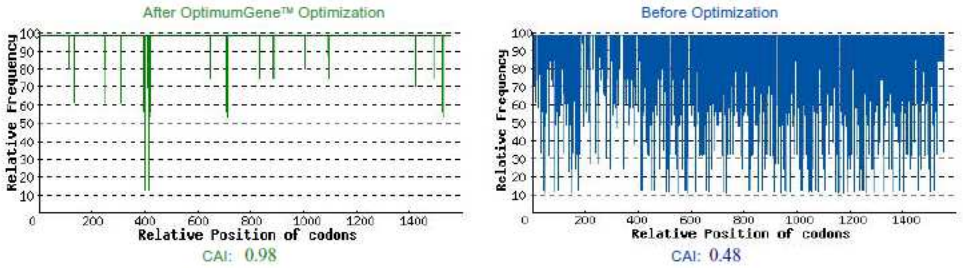
Fig.3.17: N-glycosilation sites prediction study. A) The putative N-glycosilation sites are written in blue. The N residue with higher probability to bind the sugar chains are written in red. **B)** The plot summarizes the position of N-glycosilation sites and their N-glycosilation potential.(www.cbs.dtu.dk/services/). **C) Glycosylation assay.** Twenty microliter of each reaction mix were loaded in the Western blotting. The sign (-) and the sign (+) indicate the absence or the presence of enzyme respectively.

In the IL4PE40 sequence the restriction enzymes of pPICZα multi cloning sites were removed (XhoI, XbaI, PmeI, SacI, SfiI, PstI and EcoRI) (Fig. 3.18).

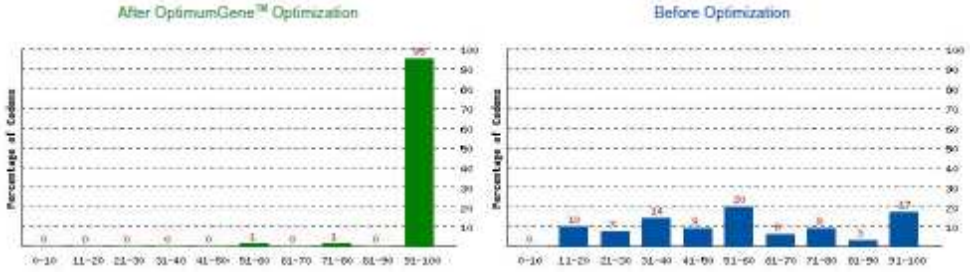
Fig. 3.18: Structure of IL4PE40 optimized.

With the sequence optimization the codon usage bias in *P. pastoris* was increased by upgrading the CAI (Codon Adaptation Index) from 0.48 to 0.98. The GC content and unfavourable peaks were optimized to prolong the half-life of the mRNA. The Stem-Loop structures, which impact on ribosomal binding and stability of mRNA, were broken.

A



B



C

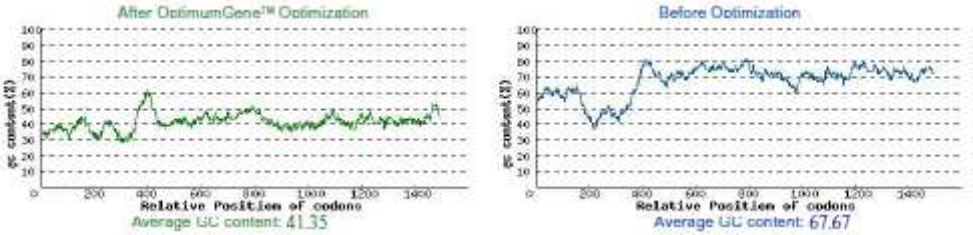


Fig. 3.19: IL4PE40 optimized: GenScript analysis. A) The distribution of codon usage frequency along the length of the gene sequence. A CAI of 1.0 is considered to be optimal in the desired expression organism, and a CAI of >0.9 is regarded as very good, in terms of high gene expression level. **B)** The percentage distribution of codons in computed codon quality groups. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism. **C)** The ideal percentage range of GC content is between 30-70%. Peaks of percentage of GC content (%GC) in a 60 bp window have been removed.

3.4.4.1.1 Screening of *P. pastoris* transformed with IL4PE40-opti construct

The IL4PE40 optimized gene (IL4PE40-opti) was provided by GeneScript inserted into the pUC57 plasmid. Therefore it was cloned in the pPICZα plasmid with the same strategy used from the non optimized gene.

Six zeocin positive clones were screened (72h of induction), and 200 µl of each supernatant were precipitated with 10% TCA and analysed in SDS-PAGE (Fig. 3.20). The clone that produced the highest amount of Immunotoxin is the number 5.

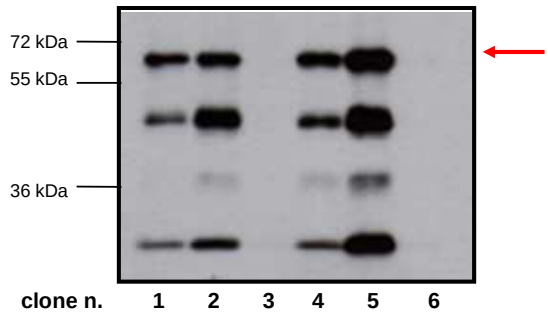
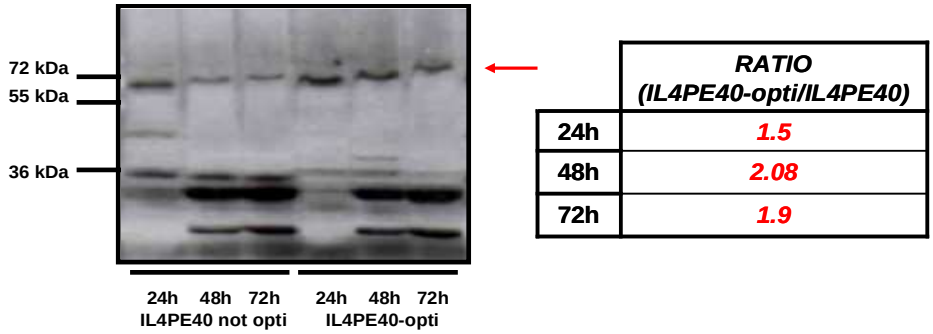


Fig. 3.20: Screening of 6 zeocin positive clones of IL4PE40-opti produced by GS115 *P. pastoris* strain. For each sample 200 µl, precipitated with 10% TCA, were analysed.

To verify if the sequence optimization increases the Immunotoxin yield, IL4PE40 and IL4PE40-opti expression was compared. (Fig. 3.21).



	RATIO (IL4PE40-opti/IL4PE40)
24h	1.5
48h	2.08
72h	1.9

Fig. 3.21: Comparison between the IL4PE40-opti and IL4PE40 production. For each sample 300µl of induced supernatant, precipitated with 10% TCA, were analysed in Western blotting. In the table at right the ratio between the amount of IL4PE40-opti and IL4PE40 are reported.

As shown in the table at right of figure 3.21, the ratio between the amount of IL4PE40-opti and IL4PE40 produced by *P. pastoris* is always greater than 1, indicating that the IL4PE40-opti amount produced at each time point is higher than IL4PE40.

3.4.4.2 IL4PE40 production optimization in *P. pastoris*

To increase the yield of protein produced in *P. pastoris*, several steps of induction condition optimization were needed. The condition changes aim at decreasing the proteolysis of recombinant protein. To identify the best condition for each variable tested, 500 µl of culture medium were precipitated with 10% of TCA, and analysed by Western blotting.

1) Time of induction. During the induction of a recombinant protein in *P. pastoris* the methanol was added at the culture every 24 h. At each time point (24h, 48h and 72h) a sample of supernatant was collected. As shown in figure 3.22 the best condition is the 24h time point, because the Immunotoxins are expressed with lower degradation.

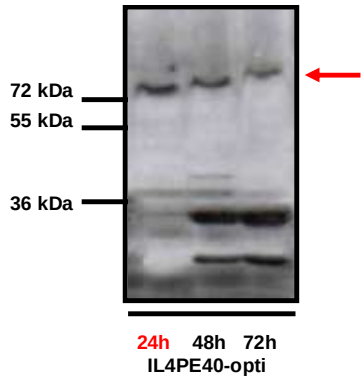


Fig. 3.22: Western blotting of different time points of IL4PE40-opti production. For each sample 300µl of supernatant, precipitated with 10% TCA, were analysed.

2) Temperature of induction. To minimize the proteolysis of recombinant proteins secreted in the culture medium the amount of Immunotoxin was analysed at three temperature: 20°C, 25°C and 30°C [Li Z., *et al.*, 2001; Curvers S., *et al.*, 2001]. As shown in figure 3.23 at 20 and 25°C the Immunotoxin was not produced, whereas at 30°C the Immunotixin is expresses.

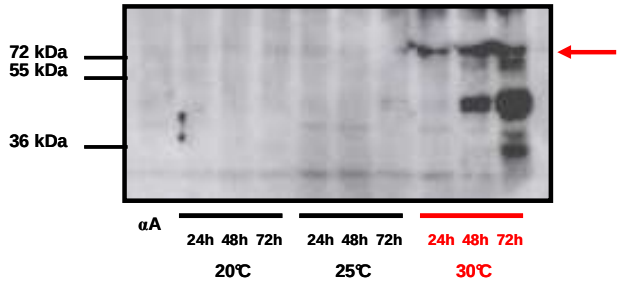


Fig. 3.23: Western blotting of IL4PE40-opti production at different temperatures. For each sample 300µl of supernatant, precipitated with 10% TCA, were analysed. Abbreviation: αA: GS115 strain transformed with pPICZαA vector (empty vector).

3) <u>Effect of cell density at the induction start.</u> The production of recombinant proteins is often proportional to the density of the host cells [Cregg J.M., *et al.*, 2000; Brierley R.A., *et al.*, 1990; Cregg J.M., *et al.*, 1995]. However, high cell density preparations are costly to prepare, increase the concentration of extracellular proteases, and may have detrimental effects on cell physiology in turn limiting the amount of desired product. As shown in figure 3.24, the best condition is the 1 OD_{600} cell density where the Immunotoxin amount is higher and the proteolysis is lower.

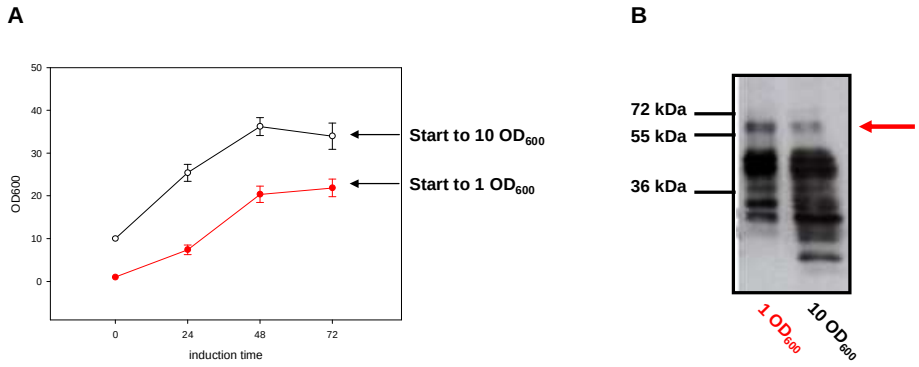


Fig. 3.24: A) Curve of growth culture starting at 1 OD_{600} or 10 OD_{600}. B) Western blotting of IL4PE40-opti production at different initial cell density. For each sample 300µl of supernatant after 48h of induction, precipitated with 10% TCA, were analysed. Abbreviation: OD: optic density.

4) <u>Presence of glycerol during the induction.</u> *P. pastoris* is a methilotrophic yeast, able to grow with the methanol as unique carbon source. Literature data [Zhang W, *et al.*, 2003] show that the presence of glycerol during the induction support the growth of the culture, with a higher cell accumulation. But sometimes, as described before for the optimization of starting cell density, the culture concentration can be proportional to the degradation level of recombinant protein. Indeed figure 3.25 shows that the whole Immunotoxin is produced only when the glycerol was not added.

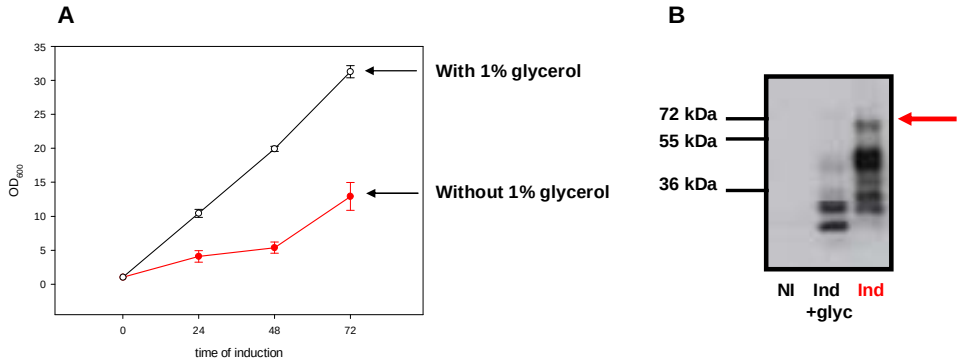


Fig. 3.25: A) Curve of growth culture of IL4PE40 induced with or without 1% glycerol. B) Western blotting of IL4PE40-opti production with or without 1% glycerol. For each sample 300µl of supernatant after 48h of induction, precipitated with 10% TCA, were analysed. Abbreviation: OD: optical density.

5) <u>Methanol concentration.</u> *P. pastoris* expression system uses the methanol both for the induction of the proteins and as the main carbon source for cell growth following induction [Cai H. *et al.*, 2009]. Generally a lower methanol concentration limits cell growth due to carbon limitations, and a higher methanol concentration is toxic because of accumulation of its metabolic products [J.M. Cregg *et al.*, 1993]. Then the methanol concentration resulting in the highest amount of expressed Immuntoxin was investigated.

The figure 3.26A shows that, after 48h of induction, a methanol feed of 0,25% (v/v) led to the highest amount of Immunotoxin, and a methanol feed of 1% (v/v) led to the lowest yield of Immunotoxin. The corresponding cell growth curves (Fig. 3.26B) during the induction phase showed that growth culture was not affected by the different inductor concentrations. This means that in the concentration range tested the influence of methanol regards only the induction pathway. In fact increasing the amount of methanol (3% and 5%) both the expression and the cell growth were inhibited (data not shown).

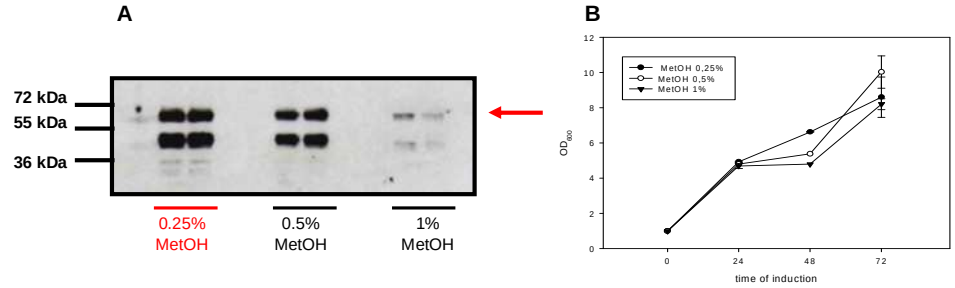


Fig. 3.26: A) Western blotting of IL4PE40-opti with different methanol concentration. For each sample 300µl of supernatant after 48h of induction, precipitated with 10% TCA, were analysed. **B) Cell growth curves of IL4PE40 induced with three different methanol concentration.** Abbreviation: MetOH: methanol.

6) <u>Medium pH.</u> Under constant pH conditions, cells can grow more efficiently [Chiruvolu V., *et al.*, 1998]. The effect of pH on cell growth and Immunotoxin expression was examined in BMMY induction medium buffered with 100 mM potassium phosphate at different pH values (5.0, 6.0, 7.0), so as to limit protease activity (Fig. 3.27)

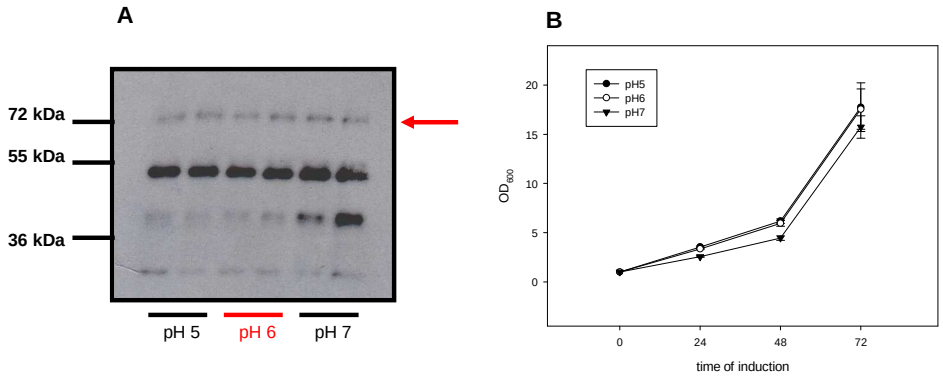


Fig. 3.27: A) Western blotting of IL4PE40-opti expresses in BMMY buffered at pH 5, 6 and 7. For each sample 300µl of supernatant after 48h of induction, precipitated with 10% TCA, were analysed. **B) Cell growth curves of ILePE40-opti expresses in BMMY buffered at pH 5, 6 and 7.**

The Immunotoxin production does not increase for any pH value, but at pH 7 the degradation level increases (Fig 3.27A). Moreover the culture growth was not affected by the pH values tested (Fig. 3.27B). The induction medium at pH 6 was therefore considered as the best condition.

7) <u>Presence of 5 mM EDTA.</u> Recombinant proteins expressed in *P. pastoris* systems have also been found to be degraded rapidly if they contain PEST sequences. These sequences include the motifs XFXRQ or QRXFX, which are known to be degraded in the lysosome [Sreekrishna K, *et al.*, 1989]. Additives such as 5 mM EDTA can also assist in stabilizing the recombinant expressed protein [Sreekrishna K, *et al.*, 1997] and often increase the yield of the expressed protein. In the IL4PE40 protein sequence a PEST sequence is present (Fig. 3.28A). But the Immunotoxin was not expressed with the EDTA in the induction medium (Fig. 3.28B), likely because the chelating action of EDTA does not support the synthesis pathway.

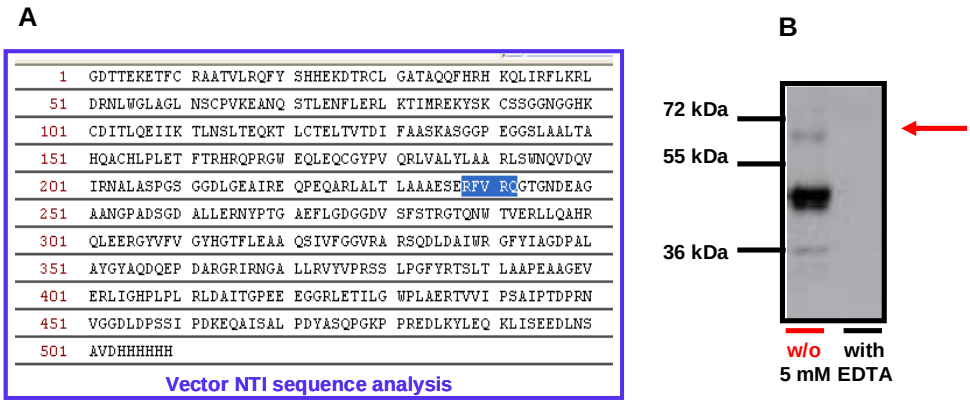


Fig.3.28: Vecton NTI software IL4PE40 sequence analysis, to identify the presence of PEST sequences (highlighted in blue). **B) Western blotting of IL4PE40-opti expresses with or without 5 mM of EDTA.** For each sample 300µl of supernatant after 48h of induction, precipitated with 10% TCA, were analysed. Abreviation: w/o: without.

8) <u>Culture conditions before induction.</u> To produce the IL4PE40 the protocol suggested by Invitrogen catalogue was used: the GS115 inoculum containing IL4PE40 gene was grown in BMGY until the logarithmic (log) phase was reached (between 2 and 6 OD_{600}), then the culture was centrifuged and resuspended in BMMY medium at the desired cell density. To produce an Immunotoxin in *P. pastoris*, Woo et al., used an alternative protocol: a single colony was grown in YPD until a saturation phase was reached (after 2 days), then the centrifuged culture was resuspended in two-thirds colture volume of BMMY medium and induced for 2 days, adding methanol every 24h. The figure 3.29 shows that the induction during the saturation phase produces more Immunotoxin than in the log phase, but with a highest degradation level. For this reason the best condition was considered the induction during the log phase of growth.

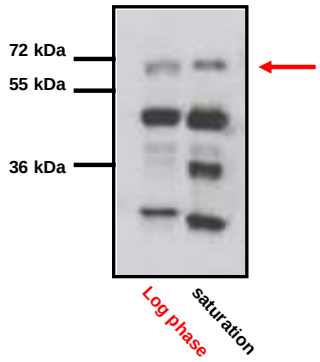


Fig 3.29: Western blotting of IL4PE40-opti expresses after inoculum during the log phase or during the saturation phase. For each sample 300µl of supernatant after 48h of induction, precipitated with 10% TCA, were analysed. Abreviation: log: logarithmic.

9) <u>Yeast strain.</u> Strains deficient in vacuolar proteases are sometimes effective at reducing protein degradation of recombinant proteins [Brierley, R. A., 1998; White, C. E., et al., 1995]. The protease-deficient strains SMD1163 (*his4 pep4*

prb1), SMD1165 (*his4 prb1*), and SMD1168 (*his4 pep4*) are particularly useful for expression of heterologous proteins in fermenter cultures because with other strains the combination of high-cell density and lysis of a small percentage of cells results in a relatively high concentration of vacuolar proteases such as proteinase A (*pep4*) and proteinase B (*prb1*).

The IL4PE40 gene was inserted in the SMD1168 genomic DNA and 14 zeocin resistant clones were screened (Fig.3.30).

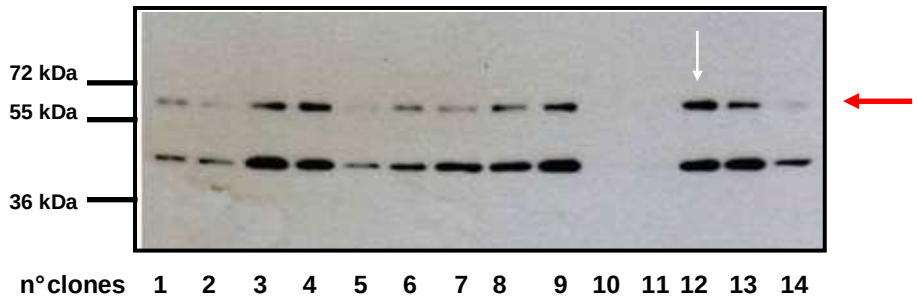


Fig. 3.30: Screening of 14 clones derived from electroporation of SMD1168 strain with pPICZalphaIL4PE40opti construct. For each sample 300µl of supernatant after 48h of induction, precipitated with 10% TCA, were analysed. The best clone is indicated with the white arrow.

The best clone was the number 12. The Immunotoxin medium-scale production was analysed using both the induction during the log phase and during the saturation phase (Fig.3.31).

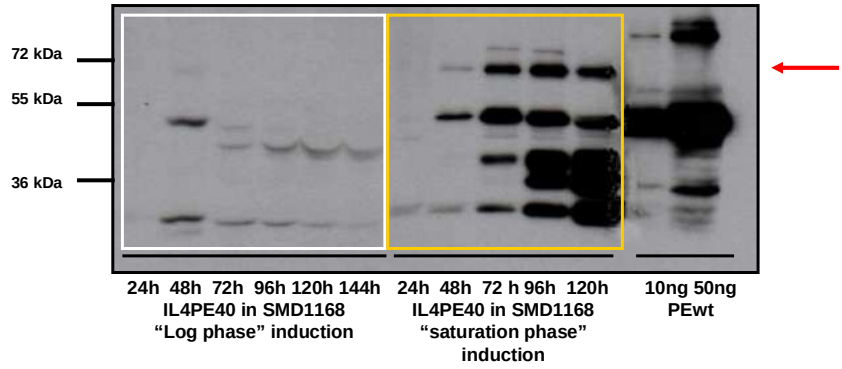


Fig. 3.31: Western blotting with SMD1168 transformed with IL4PE40-opti. Time course of IL4PE40-opti induced in "log phase" (white panel) and in "saturation phase" (yellow panel). As control 10 ng and 50 ng of PEwt were loaded. For each sample 300µl of supernatant after 48h of induction, precipitated with 10% TCA, were analysed.

As shown in figure 3.32 a higher amount of IL4PE40 was produced using the "saturation phase" protocol, even if the degradation pattern is the same derived with the GS115 strain. Comparing the intensity of IL4PE40 bands with those of

PEwt, it is possible to conclude that this *P. pastoris* protease-deficient strain is not able to produce an undegraded Immunotoxin (10 µg from 1 liter of culture) at a yield higher than that obtained with the GS115 strain (30 µg from 1 liter of culture – data not shown).

10) <u>Presence of an antifoam additive.</u> During the Immunotoxin induction the foam in the culture medium was always observed. It is possible that the interfacial foam leads to degradation of the recombinant protein [Zeev-Ben-Mordehai T., *et al.*, 2009]. To improve the Immunotoxin stability and yield an antifoam agent was added daily to the induced culture.

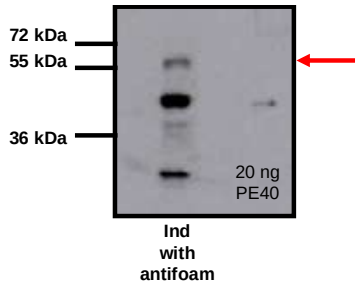


Fig. 3.32: Western blotting with IL4PE40-opti induced in BMMY medium containing an antifoam agent. The sample derived by 660µl of supernatant, after 48h of induction, precipitated with 10% TCA. As control 20 ng of PEwt were loaded. Abbreviation: Ind: Induction.

From figure 3.32 it is possible to quantify the Immunotoxin yield at 120 µg from 1 liter of culture; comparing this yield with the Immunotoxin amount produced without the antifoam (30 µg from 1 liter of culture) it is possible to conclude that the antifoam increased the yield of recombinant protein, even if the antifoam does not decrease the degradation level.

Table 3.2 shows the yield of IL4PE40 under optimized or non optimized conditions and the table 3.3 summarizes the best conditions tested for the IL4PE40-opti expression.

Strains	***Non optimized sequence***		***Optimized sequence***	
	Non optimized conditions	***Optimized conditions***	***Non optimized conditions***	***Optimized conditions***
GS115	**20 µg**	n.d.	**30 µg**	**120 µg**
SMD1168	n.d.	n.d.	**10 µg**	n.d.

Table 3.2: IL4PE40 production under optimized or non optimized conditions. Abbreviation: n.d., not determined.

	Best condition		Best condition
Time	24 h	***Medium pH***	pH 6
Temperature	30°C	***5 mM EDTA***	no
Starting cell density	1 OD_{600}	***Culture condition***	log phase
1% Glycerol	no	***Strain***	GS115
Methanol	0.25%	***Antifoam***	yes

Table 3.3: Best conditions to produce IL4PE40-opti.

3.4.5 Purification of IL4PE40-opti by affinity chromatography.

As shown before in figure 3.14A, the IL4PE40-opti gene was cloned in frame with the His_6-tag. After ammonium sulphate precipitation the culture medium was incubated 1-2 hours with the Ni-Sepharose resin. The mixture was loaded to the column, the resin was washed with the binding buffer with 20 mM imidzole and then the Immunotoxin was eluted with the same buffer containing 500 mM imidazole (Fig. 3.33) .

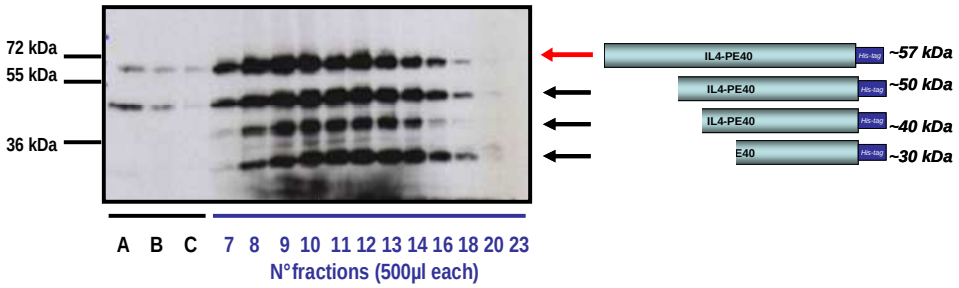


Fig. 3.33: Western blotting with the sample derived by the purification step. A: medium concentrated before binding to the resin; B: flow-through after binding; C: resin wash (20 mM imidazole); in blue: eluted fractions (500 mM imidazole). At righ of the figure, diagram representation of possible Immunotoxin fragments.

The amount of Immunotoxin produced by *P. pastoris* is low; in fact it is not possible to detect the bands corresponding to the Immunotoxin in the SDS-PAGE (Coomassie staining) (data not shown). From Western blotting analysis (fig. 3.34) it is possible to observe that in the flow-through the Immunotoxin is insted present, this likely means that the binding is not optimized; moreover the eluted fractions show 3 degradation products, as indicated at the right of figure 3.33.

As described before in the introduction (1.2.2.2.2) the native PE toxin contains a furin cleavage site in the translocation domain that releases a PE fragment of 37 kDa.

Furin-like enzymes recognize a RXXR polypeptide sequence and cleave after the last R residue.

We have hypothesized that proteases cleaving the recombinant molecules cold be present in the yeast medium.

To evaluate this hypothesis, the presence of furin-like cleavage sites was investigated by computer-based prediction (Fig.3.34).

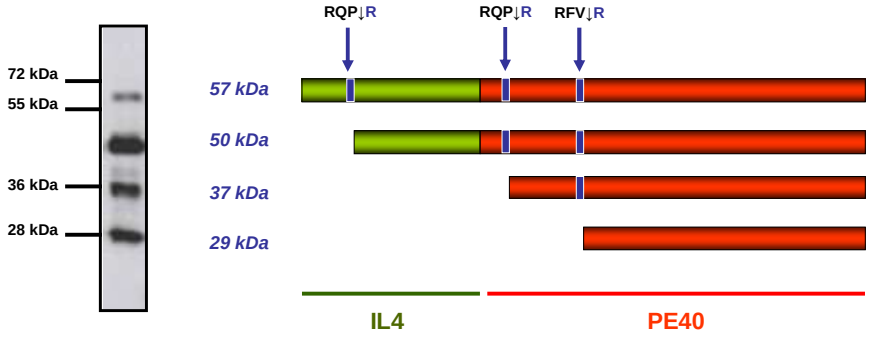


Fig. 3.34: *In silico* study of furin-like cleavage sites in the IL4PE40 sequence.

As shown in figure 3.35, the IL4PE40 protein has 3 putative furin-like cleavage sites, and the molecular weight of released fragments corresponds to the weight of degradation bands present in the Western blotting pattern.

To verify this hypothesis a cleavage test of native PE was performed (Fig. 3.35A). The native PE was incubated for 1h with: 1) PBS as a control, 2) only BMMY induction medium that is not inoculated with GS115, 3) BMMY after 48h of induction of GS115 transformed with the empty vector (αA) and 4) BMMY with 1 mM PMSF (proteases inhibitor) after 48 h of induction of αA.

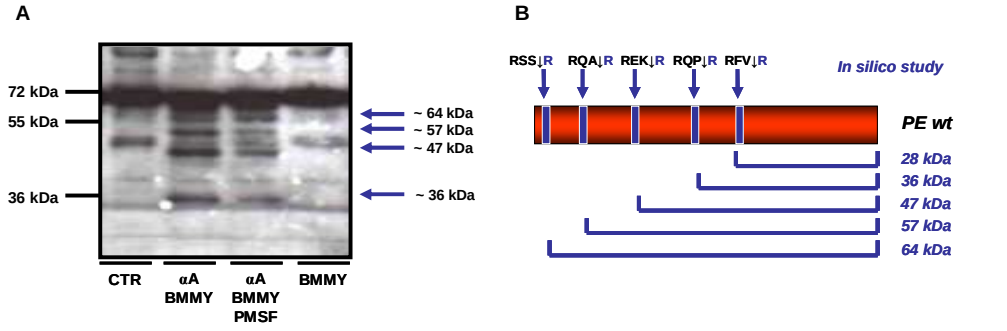


Fig. 3.35: A) Western blotting of native PE fragments derived from PE cleavage under different conditions. CTR: native PE incubated with PBS; **αA BMMY**: native PE incubated with cell-free BMMY after 48h of induction of GS115 transformed with the empty vector pPICZαA (αA); **αA BMMY PMSF:** native PE incubated with cell-free BMMY + 1 mM PMSF after 48h of induction of GS115 transformed with the αA. **BMMY:** induction medium only without *P.pastoris.* **B) In silico study of furin-like cleavage sites in the native PE sequence.**

As shown in figure 3.35A the native PE incubated with non inoculated BMMY is comparable to the PE of the control lane. This means that the agents that cleave the recombinant protein, are not present in the medium of induction, but are produced by the yeast culture. Indeed, observing the lanes of samples where the PE was incubated with αA-BMMY or αA-BMMY+PMSF, 4 bands of degradation are present. These bands are less intense in the sample with PMSF. This means that the *P. pastoris* culture may secrete the proteases cleaving the native PE; indeed if a protease inhibitor was added the degradation decreased. An *in silico* study of the native PE sequence shows 5 putative cleavage sites (Fig. 3.35B). The predicted C-terminal PE fragments have molecular weights similar to those showed in the Western blotting pattern.

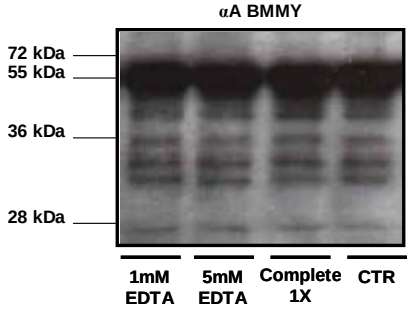


Fig. 3.36: A) Western blotting with the cleavage test of native PE. The native PE was incubated with: αA BMMY with 1 mM EDTA, αA BMMY with 5 mM EDTA, αA BMMY with inhibitors mix Complete 1X and only αA BMMY.

The cleavage test of native PE was carried out by incubating the toxin with the αA BMMY medium with 2 different concentrations of EDTA and with Complete 1X, an alternative inhibitor proteases mix (Fig. 3.36). In figure 3.36 it is possible to observe that the degradation pattern of native PE is the same in all conditions, indicating that the cleaving agents degrading the toxin are not inhibited by EDTA or by Complete.

To analyse the presence and the nature of cleaving agents 3 IL4PE40 site specific mutageneses were planned: 3 arginines (Arg^{21}, Arg^{170}, Arg^{243}) were mutated in 3 alanines using the Quik Change Multi Site-Directed Mutagenesis Kit (Stratagene).

Preliminary studies demonstrate that the mutated Immunotoxins do not show the degradation band at 37 kDa (data not shown). Further analysis will be needed to verify if the Immunotoxin is expressed without degradation products and if its cytotoxic activity is maintained.

4. DISCUSSION

This work was done to set out the optimal conditions for the expression of heterologous proteins in the methylotrofic yeast pichia pastoris, in particular to optimally express a cytotoxic macromolecular therapeutic utilizable in the management of pancreas tumors.

In this work, I subcloned the gene encoding the IL4(38-37)PE40 Immunotoxin into the pPICZα- vector, and it was expressed in *P. pastoris*. After preliminary induction the Immunotoxin was successfully secreted into the supernatant. However the amount of recombinant protein expressed was very low (20 µg from 1 liter of culture). This result however was not considered sufficient for the obtainment of Immunotoxin by the *P. pastoris* system. Several steps of optimization conditions were needed to increase the protein production (120 µg from 1 liter of culture) in the *P. pastoris* expression system.

4.1 Gene codons optimization

Many works show that the gene codon optimization increases the recombinant protein expression in *P. pastoris* [Jung Hee Woo., *et al.*, 2002; Da Teng, *et al.*, 2007; Huoqing Huang, *et al.*, 2008; Zhaorong Wei, *et al.*, 2008; Hui Wanga, *et al.*, 2008]. The IL4PE40 gene was therefore optimized. The optimization process changed not only the rare codons for the yeast, but it increased the mRNA half-life with the reduction of percerntage of GC content (from 67% to 41%) and cis-acting elements. The sequence optimization increased the Immunotoxin expression of about 1.8 fold compared with the non optimized version.

4.2 Optimization of the induction conditions

Under the optimized conditions (induction time, 24 h; temperature of induction, 30°C; initial inoculum, 1 OD_{600}; pH 6.0; 0.25% methanol concentration; induction in log phase; production with GS115 strain; presence of an antifoam agent), approximately 120 µg of protein were recovered from 1 L of the culture.

To increase the amount of recombinant proteins produced by *P. pastoris*, it was considered necessary also to decrease the proteolysis of new proteins secreted in the supernatant.

- Medium pH

X. Shi *et al.* (2003) demonstrated that several protease families were secreted from *P. pastoris* and that they were activated at different pH values. In Shi's work the media at pH 5, pH 6 and pH 8 show lower proteases activity. Since some proteins are specifically susceptible to proteases secreted in the medium of *P. pastoris*, I examined whether the pH of the culture medium would affect the IL4PE40 expression. IL4PE40 production was most efficient at pH 5.0–6.0; at pH 7 the immunotoxin was produced but with a higher level of degradation. For this reason I maintained the culture at pH 6.0, as advised by the *P. pastoris* expression protocols provided with the *P. pastoris* strains used.

- Effect of cell density at the induction start

Generally, very high cell densities are employed to produce heterologous proteins, since production is roughly proportional to cell density [J.M. Cregg, J., *et al.,* 2000; R.A. Brierley, *et al.,* 1990; J.M. Cregg, and D.R. Higgins., 1995]. With IL4PE40 expression the high cell density or the addition of glycerol as a better carbon source than methanol increase the degradation level, probably because a high amount of cells secretes more proteins, including proteases.

- Presence of EDTA

Recombinant proteins expressed in *P. pastoris* could be degraded more rapidly if they contain PEST sequences. These sequences include the motifs XFXRQ or QRXFX, which are known to be degraded in the lysosomes [Sreekrishna K., *et al.,* 1989]. The presence of 5 mM EDTA can also assist in stabilizing the recombinant expressed protein [Sreekrishna K., *et al.,* 1997] and thus increase its expressed yield. IL4PE40 contains one PEST sequence, but the EDTA addition inhibits protein production. The EDTA additive can indeed assist the degradation under certain conditions but it is also toxic to the cell culture. To overcome this problem decreased concentrations of EDTA can be tested to find the best amount that does not inhibit the protein production and that reduces Immunotoxin degradation.

- Culture conditions before induction

The IL4PE40 expression was performed as described in the protocol for *P. pastoris* handling. The yeast culture was grown in BMGY medium to reach the logaritmic phase; the cells were then centrifuged and resuspended in BMMY medium to induce the protein. Woo *et al.* (2002) produced an Immunotoxin in *P. pastoris*, using another protocol: the yeast culture was grown in YPD medium for two days to reach the saturation phase; to induce the Immunotoxin the pelleted culture was resuspended more concentrated in BMMY. Again the higher cell density leads to a higher degradation of proteins.

- Yeast strain

To decrease IL4PE40 degradation I used the SMD1168 protease deficient strain (*pep4*-). However, the use of SMD1168 did not solve the degradation problem, likely because the proteinase A was not involved in the Immunotoxin degradation. Moreover the yield of Immunotoxin in SMD1168 was lower than in the GS115 strain, as described in other works showing a lower ability of the proteases deficient strains to produce high amounts of recombinant proteins [Cereghino J.L and Cereghino G.F.L., *Methods in Molecular Biology,* vol. 389: *Pichia Protocols*, Second Edition].

- Presence of an antifoam additive

The foam produced during the culture agitation can unfold proteins found in the medium. To decrease the loss of active Immunotoxin secreted into the induction medium an antifoam agent was added daily to reduce the air/liquid interfacial foam area. This increased the amount of expressed Immunotoxin of about 10-fold.

- Methanol concentration

In the case of the *P. pastoris* expression system, methanol is used both for the induction of the protein expression and as the main carbon source for cell growth following induction. Hence, an optimal concentration of methanol is an important factor that affects the yield of the heterologous protein produced by *P. pastoris*. According to the manual of the *P. pastoris* expression system provided by

Invitrogen, 0.5% of methanol is recommended to induce protein expression. In our experiment, I observed that IL4PE40 expression was induced by a daily addition of methanol 0.25% to 1.0% final concentration. However, the maximal accumulation of immunotoxin in the supernatant was obtained at a methanol concentration of 0.25%. The protein was not induced by 3.0% or 5.0% methanol and the cell growth was halted by higher concentrations of methanol. Then the IL4PE40 production was induced by the lowest percentage of methanol.

- Time and temperature of induction

The time and temperature of induction are other variables that may reduce the proteolysis. In fact it is important to find an equilibrium between the highest amount of produced protein and the lowest degradation level. To overcome this problem it is possible to use the medium substitution procedure during the protein induction (reiterative induction). In our case the best expression of IL4PE40 (highest amount, lowest degradation) was at 24h of induction. Since no tested condition prevents the proteolysis at 48h of induction, the reiterative induction of the same cell preparation every 24h could partially solve the degradation problem.

4.3 Toxicity of IL4PE40 in the host cells

Observing the results of the induction optimization, the best yields of Immunotoxin were obtained under the conditions that slow down the cellular growth. In fact, the high cell concentration seems to lead to a decreased Immunotoxin production. This feature disagrees with the results obtained from the optimization of other non toxic proteins where the best yield was obtained in the high density cultures. However the Immunotoxin yield after optimization is against very low.

IL4PE40 is a molecule which is toxic also to the yeast system, because the catalytic domain of PE is able to ADP-ribosylate the EF-2 of all eukaryotic cells. It cannot be excluded that the Immunotoxins or the toxin fragments reach the yeast cytosol ribosomes. The Immunotoxins could therefore translocate to the cytosol from the endoplasmic reticulum or re-enter the cells after secretion.

The C-terminal REDLK endoplasmic reticulum retention sequence of PE40 could retain the Immunotoxin in the vesicles pathway, decreasing the secreted

protein and increasing the possibility of toxin translocation to the cytosol. But the C-terminal his_6-tag of the recombinant Immunotoxin likely prevents the REDLK recognition by the vesicle receptors.

The yeast expression system is a good host to produce the mammalian proteins because it lacks the mammalian receptors such as IL4R. For this reason the receptor-mediated endocytosis of the Immunotoxin is unlikely. However it is possible that non specific pinocytosis events may lead to the internalization of the Immunotoxins which would then reach the endoplasmic reticulum and subsequently the cytosolic ribosomes.

To overcome this problem with a DT-based immunotoxin Woo and co-workers chose to express the catalytic A chain of Diphtheria toxin (DT) within the cytosolic compartment of *P. pastoris*. The colonies that grow after induction have a DT resistant EF-2. To evaluate if the IL4PE40 reaches the cytosol during the induction, leading to inactivation of protein synthesis with consequent low production of secreted Immunotoxin, I analysed the different yeast compartments by Western blotting. The Immunotoxin was accumulated mostly in the supernatant. Traces of Immunotoxins were present also in the insoluble fraction of the cytosol (data not shown). The insoluble fraction corresponds to the inactive and unfolded inclusion bodies of *E. coli*. Since the PE is a powerful toxin, it is possible that an amount undetectable by Western blotting reaches the cytosol and inhibits the protein sinthesys. For this reason it could be interesting to analyze the Immunotoxin expression in a PE resistant strain.

4..4 Other assay conditions

Other factors which I have not yet tested might affect the yield of heterologous proteins produced in *P. pastoris*.

- Medium composition

Several reports indicate that medium composition influences heterologous protein expression in yeast by affecting cell growth and viability [Kang H.A., *et al.*, 2000; Shiba Y., *et al.*, 1998; Chen D.C., *et al.*, 2000] or the secretion of extracellular proteases [Sreekrishna K., *et al.*, 1997; Chung B.H., *et al.*, 1997]. Shi X. and co-workers [Shi X., *et al.*, 2003], demonstrated that the addition of 2% of

casamino acids or of 0.4 M L-arginine in the BMMY medium increased the protein accumulation. Particularly L-arginine addition decreased cell growth, but increased protein accumulation. The study of this condition could be interesting in our case, where the amount of IL4PE40 was higher when the cell growth was lower.

Propagation of *E. coli* under high osmotic pressures was reported to increase production of soluble, heterologous enzyme several hundred fold [Blackwell J.R., *et al.,* 1991]. Culturing *P. pastoris* in hypertonic media containing either 0.35 M potassium acetate or 0.35 M sodium chloride prior to induction increases protein production [Shi X., *et al.*, 2003]. During the induction the hypertonicity of BMMY medium led to rapid cell lysis and little protein production [Shi X., *et al.,* 2003]. Moreover, sorbitol and alanine in the BMMY medium were reported to be adequate carbon sources, supporting cell growth while not suppressing the AOX promoter [Sreekrishna K., *et al.,* 1997].

- Presence of the protease inhibitors during the induction

The addition of PMSF (mix of protease inhibitors) in the induction culture medium could decrease the protein degradation and then increase its yield [Woo JH, *et al.*, 2004]. I added 1 mM PMSF during the immunotoxin induction but the yield of protein dramatically decreased (< 5µg from 1 liter of culture) and the degradation problem was not solved (data not shown). Also in this case it will be necessary to test different PMFS concentrations to find the equilibrium between the proteases inhibition and the toxicity of PMSF for the cultures cells.

- Intracellular expression

The toxicity of IL4PE40 does not allow an efficient intracellular expression. For this reason I have chosen the pPICZα expression vector driving the recombinant proteins through the secretion pathway thanks to the α-mating factor signal sequence. To direct the proteins into the secretion pathway of *P. pastoris*, several signal sequences can be chosen.

- Signal sequences for the secretion pathway

IL4PE40 was expressed only with the α-mating factor signal sequence. It is possible to test the secretion ability of other yeast signal sequences, such as PHO

(acid phosphatase signal sequence) or SUC2 (invertase signal sequence). The IL4 immunotoxin carrier portion is a secretory molecule, therefore it has a native secretory sequence. The IL4 moiety could be cloned in frame with its native signal sequence to test the yeast ability to drive the protein through the secretory pathway. Indeed some proteins were produced successfully with the native secretory sequence [Barr, K.A, *et al.,* 1992; Ridder, R., *et al.,* 1995].

- Promoters

IL4PE40 gene was cloned into the pPICZα plasmid containing the AOX1 promoter and terminator. Although the AOX1 promoter has been successfully used to express several foreign genes, to optimize the Immunotoxin production it is possible to test the protein expression using alternative promoters, such as GAP, FLD1, PEX8, and YPT1.

The GAP gene promoter provides strong constitutive expression on glucose. The advantage of using the GAP promoter is that methanol is not required for induction, nor is it necessary to shift cultures from one carbon source to another. However, since this is constitutively expressed, it is not a good choice for the production of proteins that are potentially toxic to the yeast such as an Immunotoxin.

The FLD1 gene encodes a glutathione-dependent formaldehyde dehydrogenase, a key enzyme required for the metabolism of certain methylated amines as nitrogen sources and methanol as a carbon source [Shen S., *et al.*, 1998]. The FLD1 promoter can be induced with either methanol as a sole carbon source (and ammonium sulphate as a nitrogen source) or methylamine as a sole nitrogen source (and glucose as a carbon source) and than offers the flexibility to induce high levels of expression using either methanol or methylamine, an inexpensive nontoxic nitrogen source.

For certain foreign genes, the high level of expression from AOX1 promoter may overwhelm the post-translational machinery of the cell, causing a significant proportion of foreign protein to be misfolded, unprocessed, or mislocalized [Thill G.P., *et al.,* 1990; Brierley R.A., 1998]. For some proteins such as on Immunotoxin, moderately expressing promoters could be desirable. The *P. pastoris* PEX8 and YPT1 promoters may be used. The PEX8 gene encodes a

peroxisomal matrix protein that is essential for peroxisome biogenesis [Liu H., *et al.*, 1995]. It is expressed at a low but significant level on glucose and it is induced modestly when the cells are shifted to methanol. The YPT1 gene encodes a GTPase involved in secretion, and its promoter provides a low but constitutive level of expression in media containing either glucose, methanol, or mannitol as carbon sources [Sears I.B., *et al.*, 1998].

- Yeast strains

The IL4PE40 construct was produced by two different host strains: GS115 and SMD1168 (Mut^+). The Mut^+ strains grow on methanol at the wild-type rate. However, two other types of host strains are available which vary with regard to their ability to utilize methanol because of deletions in one or both AOX genes. Strains with AOX mutations are sometimes better producers of foreign proteins than wild-type strains [Tschopp J.F., *et al.*, 1987; Cregg J.M., *et al.*, 1987; Chiruvolu V., *et al.*, 1997]. Additionally, these strains do not require the large amounts of methanol routinely used for Mut^+ strains. KM71 is a strain where AOX1 has been partially deleted and replaced with the *S. cerevisiae* ARG4 gene [Cregg J.M. and Madden K.R., 1987]. Since the strain must rely on the weaker AOX2 for methanol metabolism, it grows slowly on this carbon source (Mut^s, methanol utilization slow phenotype). Another strain, MC100-3, is deleted for both AOX genes and is totally unable to grow on methanol (Mut^-, methanol utilization minus phenotype) [Cregg J.M., *et al.*, 1998].

The SMD1168 strain used to produce IL4PE40 is a protease-deficient strain that lacks of PEP4 vacuolar protease. As previously described in the *Results* chapter, Immunotoxin production with this *P. pastoris* strain does not solve the degradation problem; moreover the yield of protein decreases. In addition to lower viability, it possesses a slower growth rate and it is more difficult to transform. SMD1163 strain is another protease-deficient strain that lacks of PEP4 and PRB1 proteases. It is possible that this strain would decrease the IL4PE40 degradation level. Therefore, the use of protease-deficient strains is only recommended in situations where other measures to reduce proteolysis yield unsatisfactory results.

Another possibility to increased the IL4PE40 yield is that to created PE resistant mutant strains using the strategy of Liu Y.Y. *et al.* (2003). The

eukaryotic elongation factor-2 (EF-2) contains a post-translationally modified histidine residue, known as diphthamide, which is the specific ADP-ribosylation target of diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A [Zhang Y *et al.,* 2008]. Introducing the specific mutations in the EF-2 and inserting by homologous recombination the mutated gene into the yeast genome, it is possible to select PE resistant clones to produce higher amounts of the PE-based Immunotoxins.

4.5 The cleavage of IL4PE40 by *P. pastoris*

In 2005, Gurkan C. and Ellan D. summarized the different bacterial toxins, or their portions, that were successfully express by *P. pastoris*. They report the ability of *P. pastoris* to produce the tetanus toxin fragment C (TeNT) and the heavy fragment C of the botulinum neurotoxin (BoNT). In both TeNT and the BoNT variants, the carboxyl-terminal domain of the heavy chain (HC) is non-toxic and associated with binding to specific receptors present on the target nerve cells, and since it is antigenic, it has been exclusively used for the development of vaccines [Smith LA, *et al.,* 1998; Byrne MP and Smith LA., 2000]. *P. pastoris* also proved very useful in the development of vaccines for the heat-labile enterotoxin (LT) of *E. coli* and the cholera toxin (CT) of *Vibrio cholerae* [Harakuni T, *et al.,* 2005; Fingerut E., *et al.,* 2005]. As previously described in the *Introduction* (Table1.12), *P. pastoris* is able to produce the fusion proteins with bacterial toxic protein such as DT and Cyt2Aa1 (δ-endotoxin from the *Bacillus thuringiensis*), but PE-based fusion proteins produced by *P. pastoris* were not described in the literature.

4.5.1 Degradation

After IL4PE40 affinity chromatography purification from *P. pastoris* supernatant, the eluted fractions show several degradation products bearing the his_6-tag (Fig. 3.33 of *Results*). Therefore, I hypothesized the presence of agents cleaving the proteins secreted by yeast.

The native PE has a furin-cleavage site that releases a C-terminal 37 kDa fragment (II and III domains). This cleavage is essential for the toxin enzymatic activity. The IL4PE40 degradation pattern shows a 37 kDa band. Therefore I

searched the furin-cleavage sites in the IL4PE40 protein sequence. The minimal cleavage-site recognized from furin-like enzymes is RXXR. In the immunotoxin sequence three furin-like cleavage sites are present. Moreover the released C-terminal fragments have a molecular weight comparable to that of the degradated bands (Fig. 3.34). To confirm this hypothesis I carried out the same *in silico* study of furin-like cleavage sites in the native PE (Fig. 3.35B). Also the native toxin has the cut sites for the furin-like enzymes releasing five C-terminal fragments with the molecular weights comparable to that of degradated bands pointed out in the Western blotting of figure 3.35. The cleavage test reported in figure 3.35, shows that the PMSF inhibitor decreases the degradation level. Moreover, the second cleavage test (Fig. 3.36) shows that 5 mM EDTA does not inhibit the protein cleavage. The PMSF inhibits the serin proteases, but not the furin-like proteases, whereas the EDTA inhibits the furin-like proteases but not the serine proteases [Chiron M.F. *et al.*, 1994].

The serine proteases are enzymes cutting peptide bonds in which one of the amino acids at the active site is serine. Serine proteases are grouped into families that share structural homology such as the chymotrypsin-like. Interestingly chymotrypsin-like enzyme is the trypsin that is responsible for cleaving peptide bonds following a positively-charged amino acid residues (arginines and lysines) [Jesper V., *et al.*, 2004]. As previously describe, the furin-like cleavage site contains two arginines; it is possible that these residues are recognized by the serine proteases such as trypsin. *P. pastoris*‘s pro-proteins, activated by the cut of trypsin-like cleavage sequence, were described in several works and confirm the existence of trypsin-like enzymes in the yeast [Manjithaya R., *et al.*, 2010; Reverter D.,et al., 1998; Ohi H. et al., 1996]. As previously described in the optimization of Immunotoxin production, L-arginine addition in the induction medium is able to increase the protein accumulation. In fact it is able to inhibit the extracellular trypsin-like proteases [Chung B.H., *et al.*, 1997].

Shi X. and colleagues (2003) described the proteases activity in the *P. pastoris* supernatants (Fig. 4.1). The induction medium was buffered with the potassium phosphate at different pH values.

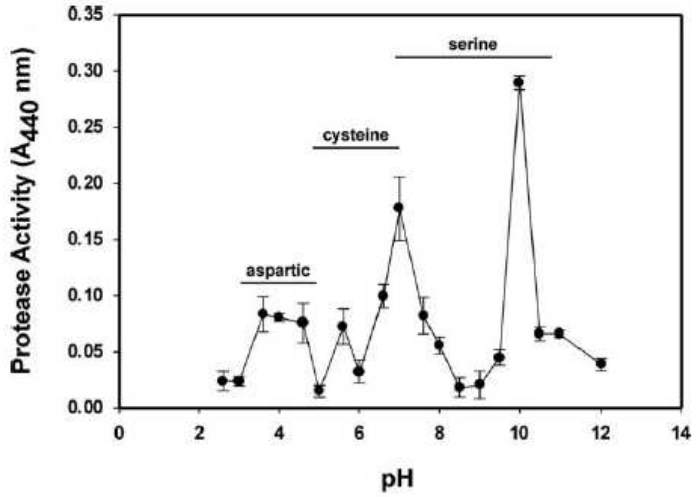


Fig. 4.1: Protease activities from *P. pastoris* culture supernatants. The graphic shows protease activity toward azoalbumin relative to the pH of medium. [Modified by Shi X., et al., 2003]

The figure 4.1 shows three proteases typologies active at different pH values of culture supernatant. The IL4PE40 optimal production was at pH 6. When IL4PE40 was induced in the BMMY medium at pH 7 the proteolysis increased (Fig. 3.27). We supposed that the cleavage of serine proteases (active between pH 7 and pH 10) is supported by the cysteine proteases activity (active between pH 5 and pH 7). But if the IL4PE40 protein sequence was inserted in the "PeptideCutter" tool (http://www.expasy.ch/tools/peptidecutter/) the cysteine protease cleavage sites were not identified.

The recombinant proteins secreted by *P. pastoris* were synthesized in frame with the α-mating factor signal sequence (αMF). Thanks to αMF, the new protein was driven through the secretory pathway and than released in the culture medium. Kex2 is a trans-Golgi membrane serine protease, specific for the cleavage of basic amino acids. The αMF cleavage allows the release of recombinant protein in the extracellular environment. The figure 4.2 shows the Kex2 cleavage site contained in the IL4PE40 protein sequence ($K^{47}R^{48}$) that released a 50 kDa fragment.

Fig. 4.2: Kex2 cleavage sequence inside of IL4PE40.

Some time, after 24h of induction it is possible to observe a 50 kDa degradation product in Western blotting (Fig. 3.21). It can be supposed that Kex2 can contribute to the proteolysis of IL4PE40 before the protein reaches the culture medium [Werten M.W.T. and de Wolf F.A., 2005].

To characterized better the nature of proteases that cut IL4PE40, several cleavage tests with single and specific protease inhibitors will be need.

4.5.2 Preventing the IL4PE40 degradation

After the native PE cleavage tests and the *in silico* studies of toxin and immunotoxin, I devised three mutageneses to change three IL4PE40 Arg residues (Arg^{21}, Arg^{170}, Arg^{243}) to alanine. However these mutageneses could inactivate the Immunotoxin; particularly the Arg^{170} that is responsible for the release of the translocation domain is essential for the cytotoxicity of the protein. Chiron M.F *et al.* (1994) reported that the arginine-to-glycine PE mutant (Agr^{279} of PE furin-like cleavage site) was not cleaved by furin-like proteases and it was approximatelly 500-fold less active than the native toxin. However the lack of cleavage of PE40 from the IL4 carrier may not inhibit the Immunotoxin ability to intoxicate the treated cells. Indeed some SAP- and PAP-based Immunotoxins have not a cleavage site that releases the toxic portion, but equally they are able to kill the target cells [Lombardi A, *et al.*, 2010; Flavell DJ, *et al.*, 2001; Schlick J, *et al.*, 2000].

The IL4PE40 mutagenesis Arg^{243}-to-Ala is inside the catalytic domain of PE40. The residues essential to the native PE toxic action are: His^{440}, Arg^{458}, Gln^{460}, Glu^{553} [Xiang Y., *et al.*, 1995; Li M., *et al.*, 1996]. The native PE Arg^{458} correspond to the Arg^{349} of Immunotoxin, therefore the IL4PE40 Arg^{243} mutagenesis would not inhibit the toxin ADP-ribosylation activity.

The characterization of the *P. pastoris* proteases involved in the immunotoxin cleavage could lead to active and proteases resistant PE40 development.

If I will not succed in the generation of proteases-resistant PE40, I will have in any case demonstrated that it is possible to express a full-length PE based immunotoxin in the *P. pastoris* supernatant, although so far only at a low yield.

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