IV- Treball 1. Comparative Expression of potato carboxypeptidase inhibitor in *Pichia pastoris* and *Escherichia coli*

IV.A   Introduction

Potato carboxypeptidase inhibitor (PCI) is a 39 residue globular protein that competitively inhibits several metallocarboxypeptidases (Hass and Ryan, 1981). It has a 27 residue central core with 3 disulfide bridges that forms a T-Knot scaffold, also found in serine protease inhibitors from the squash family and in the epidermal growth factor (EGF) superfamily of growth factors.

In recent years, there has been an increasing interest in the role of protease inhibitors in medicine, agriculture and nutrition. Since the biological actions of most proteases are under the control of their inhibitors, these molecules present many biotechnological applications. PCI is a plant protease inhibitor and its biological function is probably the inhibition of insect digestive metallocarboxypeptidases as part of the defense system of the potato plant against insects attack (Hass and Ryan, 1981); therefore, it is a potential candidate for plant protection against insects by genetic engineering. It has also been observed that i*n vivo* coadministration of PCI and tissue-plasminogen activator (tPA) significantly improves tPA-induced thrombolysis in rabbits and may potentially be a very effective adjunct to tPA, improving the thrombolytic therapy (Nagashima et al, 2000; Klement et al, 1999). Recently, we have also shown that PCI possesses anti-tumoral properties, related to its structural similarity to EGF and other growth factors (Blanco-Aparicio et al, 1998).

To study these possible functions of PCI, many mutant forms of it have been designed, but a powerful expression system is needed to express and purify them in a short time. Previously, a synthetic gene for PCI was constructed and cloned in the pIN3-OmpAIII plasmid, giving the vector pIMAM3 (Molina et al, 1992), but the yield of recombinant protein in flask was low (1-2 mg/liter). Here, we report the cloning, expression and purification of PCI in the methylotrophic yeast *Pichia pastoris* and in *Escherichia coli* with and without a His-tag.

IV.B.1   Construction of a Yeast Expression Vector and transformation

PCI cloned into the NcoI and EcoRI site of the pUCBM21 vector was used as a template for PCR amplification using a Taq polymerase (Life Technologies). The 5' primer used in the PCR was 5'TTCGGCTCGAGAAAAGAGAACAGCACG3' and it contained a XhoI restriction site and a sequence coding for the cleavage signal of the *Saccharomyces cerevisiae* α-factor secretion signal. The 3' primer was the Forward Universal Primer: 3'TGACCGGCAGCAAAATG5'. The amplified fragment was ligated in pGEM Tvector (Promega) and transformed into *E.coli* Xl1 Blue cells. Both, plasmid preparation and pPIC9 vector, were digested with XhoI and EcoRI (Roche) and ligated with

T4 DNA ligase (Roche). The constructed vector -pPIC9-PCI- contains an open reading frame coding for the *S. cerevisiae* α-factor secretion signal and for the PCI protein, which was confirmed by dideoxy chain termination sequencing. It was linearized with BglII and transformed into *P.pastoris* strain GS115 using the spheroplast method as described by Invitrogen protocol.

Since BglII digestion of pPCI9-PCI favors recombination at the alcohol oxidase gene AOX1, the methanol utilization phenotype (*mut^s/mut^+*) of 64 transformants were characterized for the presence of the AOX1 gene by the ability to grow on medium with methanol as the only carbon source.

IV.B.2 Selection of High Expressors and Large-Scale Expression of PCI in Yeast

20 clones were tested for expression of PCI using 2-ml cultures in 10-ml tubes. The clone with the highest level of PCI expression as determined by carboxypeptidase A (CPA) inhibition activity was used for further expression studies.

The selected transformant was pre-grown at 30ºC with vigorous shaking -300 rpm- in 2-liter baffled flasks containing 0.5 L BMGY (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, $4\cdot 10^{-5}$ % biotin, 1% glycerol, 100 mM K-phosphate, pH 6.00) for two days. The culture was centrifuged and the cell pellet was resuspended in 150 ml BMMY (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, $4\cdot 10^{-5}$ % biotin, 1% methanol, 100 mM K-phosphate and pH 6.00). The cultures were grown for 48 h at 30ºC, with addition of 1% methanol every 24 h to maintain induction. Secretion of PCI into the medium was determined by CPA inhibitory activity.

IV.B.3 Construction of a Bacterial Expression Vector: Generation of pIN3OmpAIII-HisTagPCI

The PCI gene cloned into pIN3OmpAIII vector -pIMAM3- was used as a template for PCR amplification using a Taq polimerase (Life Technologies)(Ghrayeb et al, 1984; Molina et al, 1992). The 5' primer used in the PCR was 5'ATAGAATTCATCATCATCATCATCATCGTGAACA GCACGCGGAT3', that contained an EcoRI restriction site, a region coding for 6 His and 1 Arg. The 3' primer was 3'ATGCAACCAATTATCCAGCTGAGCTCTCTAGATTA5'. The purified PCR product was cloned into pGEM T easy Vector (Promega) and transformed into *E.coli* XL1 Blue cells. Both recombinant plasmid preparation and pIN3OmpAIII vector were digested with EcoRI (Roche), ligated using T4 DNA ligase (Roche) to obtain pIN3OmpAIII-HisTagPCI and transformed into *E.coli* XL1 Blue cells. To identify colonies with the correct gene orientation, digestion of several recombinant plasmid preparations were performed using HindIII and BamHI (Roche) separately. Both enzymes present a restriction side inside the His-Tag-PCI gene and another one in the vector. Comparing the length of the restricted fragments, a colony with the right fragment combination was chosen and its

sequence was confirmed by dideoxy chain termination sequencing and was transformed into *E.coli* MC1061 cells.

IV.B.4 Construction of a Bacterial Expression Vector: Generation of pBAT4-PCI

The PCI gene cloned into pIN3OmpAIII vector was used as a template for PCR amplification using a Taq polimerase (Life technologies). The 5' primer was 5'TACCATGGCGCACGAT AACAGCTTCTA3', which contained an EcoRI restriction site. The 3' primer used was 3'GACGCCGGGCATGCAACCAATTATCGAGCTCACGT5' and contained a XhoI restriction site. Using these primers a PCR fragment was generated coding for the signal peptide OmpA, the PCI gene and the two restriction sites. After purification of the PCR product, the pBAT4 vector -which derives from the pET vectors (Studier i col, 1999)- and the amplified PCR fragment were digested with XhoI and EcoRI (Roche) and ligated with T4 DNA ligase (Roche) to obtain pBAT4-PCI, that was first transformed into Xl1 Blue *E.coli* cells. The sequence was confirmed by dideoxy chain termination sequencing and afterwards the construction was transformed into *E.coli* BL21 (DE3) cells.

IV.B.5 Screening for High-Expressing Clones and Large-Scale Expression of PCI in Bacteria

To test the effect of several parameters on PCI expression level and to determine the highest expressing clones, analytical recombinant expressions were performed. *E.coli* transfomants were cultured at 37ºC in 1 ml of Luria-Bertani (LB) medium containing 50 µg/ml of ampicillin for 14 h and then 250 µL aliquots were subcultured in 250 ml of minimal media (1liter of minimal media contains 100 ml 10xM9 salts, 1 ml SO_4Mg 1M, 3 ml Cl_2Ca 0.1 M, 1 ml Tiamine 1 M, 6.25 ml glycerol 80%, 10 ml cas aas 20% and 1ml of oligoelements solution. Such a solution contained $SO_4Fe.7H_20$ 40 mg/l, $SO_4Mn.H_20$ 10 mg/l, $Cl_2Co.6 H_20$ 4 mg/l, $SO_4Zn.7H_20$ 2 mg/l, MoO_4Na_2 2 mg/l, $Cl_2Cu.2H_20$ 1 mg/l, Cl_3Al 1 mg/l and Bo_3H_3 0.5 mg/l). The media was suplemented with100 µg/ml of ampicillin. When the cell density at 550 nm was around 0.7, IPTG was added to a final concentration of 0.2 mM in the case of pBAT4-PCI, and 1 mM in the case of pIN3OmpAIII-HisTagPCI, and the cultures were shaken at 37ºC for 16-20 h.

To analyze the levels of PCI in the analytical expressions, 10 ml of the final culture media were centrifuged at 12000 g for 5 min in a microcentrifuge and the supernatants were purified using a Sep Pak C_{18} (Waters) cartridge. The protein was eluted in 3 ml of 30% isopropanol, the organic solvent was evaporated and the sample was acidified with trifluoroacetic acid (TFA) and analyzed by reverse phase-high pressure liquid cromatography (RP-HPLC) on a Nova-Pak C_{18} 3.9x150 mm column (Waters). For large-scale expression in flask, fresh *E.coli* transformants were cultured in 50-ml tubes containing 5 ml LB medium and 50 µg/ml of ampicillin overnight at 37ºC. The grown media were

centrifuged and the cell pellets were resuspended in 2-liter flasks containing 400 ml of minimal salt media and 100 µg/ml of ampicillin. When the optical density at 550 nm reached 0.7, IPTG was added and the cultures were shaken at 37ºC for 16-20h.

IV.B.6   Periplasmic Extraction in Bacteria

The cell culture was centrifuged at 4200 g for 25 min and the cell pellet was resuspended in 1/5 of the initial volume with cold periplasmic buffer I (1liter contains 200 g sacarose, 24.2 g Tris.HCl, 37.2 g EDTA, pH 9.00). It was shaken for 10 min on ice, centrifuged at 9000g for 15 min at 4ºC and resuspended in the same volume of periplasmic buffer II (1liter contains 2.4 g Tris.HCl, pH 7.50). It was further shaken for 10 min on ice and centrifuged at 9000 g for 15 min at 4ºC. The supernatant corresponded to the periplasmic fraction.

IV.B.7   Purification of PCI

PCI was purified from the culture medium by a solid phase extraction using a hydrophobic Sep Pak C_{18} (Waters) cartridge, which was eluted with 30% acetonitrile. Acetonitrile was evaporated and the sample was submitted to a chromatography on a TSK DEAE-5PW 21.5x150 mm anionic exchange column (Toyo-Soda). Afterwards the protein was purified by RP-HPLC, on a C_4 0.46x25 cm, 5 µm Protein column.(Vydac). The conditions used were: solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA, and the gradient was 20%-40% B in 30 minutes. The final yield of protein was determined by its optical density at 280nm ($E_{0.1\%} = 3.0$) (Hass and Ryan, 1981).

IV.B.8   CPA inhibitory activity

The general assay for carboxypeptidase inhibition was carried out using the substrate hipuryl-L-phenylalanine and the buffer was 20 mM Tris.HCl, 0.5 M NaCl, pH 7.50. To 970 µl of substrate, 20 µl of the sample or control medium and 10 µl of bovine CPA (Sigma) at 0.02 mg/ml were added and the absorbance change at 254 nm was measured during 2 minutes. The slope of the assay corresponded to v_o (with control medium) or v_i. The percentage of inhibition was calculated according to:

$$[(v_o - v_i)/ v_o] \cdot 100$$

IV.B.9   Mass Spectrometry analysis

Molecular mass was determined by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) on a Bruker-Biflex Tof mass spectrometer. Ionization was accomplished with a 337 nm pulsed nitrogen laser and spectra were acquired in the linear positive ion

mode, using a 19 kv acceleration voltage. Samples were prepared mixing equal volumes of the protein solution and of a saturated solution of sinapic acid (10 mg/ml), used as a matrix, in aqueous 30% acetonitrile with 0.1% TFA.

IV.B.9.1 Purification Protocol for MS Samples

5 mg of C_{18} resin (Bondapak, Waters) were placed in a 500-µl microcentrifuge tube. 50 µl of acetonitrile were added, the mixture was vortexed and centrifuged at 15000 g for 30 s. Acetonitrile was removed and 150 µl of deionized water were added, mixed by vortexing and centrifuged at 15000 g for 30 s. Water was removed and the sample was added (200-500 µl), mixed by vortexing and centrifuged at 15000 g for 30 s. The sample was removed and the resin was rinsed twice with 500 µl of deionized water, mixing by vortexing and centrifuging at 15000 g for 30 s. Water was removed carefully and the proteins bound to the resin were eluted with 10 µl of 50% isopropanol, 0.1% TFA. After vortexing and centrifuging at 15000 g for 30 s, the supernatant of the last step was used for MALDI-TOF MS analysis (Villanueva et al, 2001).

IV.C   Results

IV.C.1   Expression of PCI by Pichia pastoris

To produce PCI in the methylotrophic yeast *P.pastoris* the expression vector pPIC9-PCI, with an open reading frame coding for the *S.cerevisiae* α-factor secretion signal followed by mature PCI, was transformed into the strain GS115. The initial test was the characterization of the methanol-utilization (mut) phenotype of these strains. Both, mut^s (containing no AOX1 gene) and mut^+ (containing AOX1 gene) transformants were identified. Out of 64 colonies, 15 were determined to be mut^s and 49 to be mut^+. The expression of PCI was studied by enzymatic assay on the crude supernatants of 20 colonies (7 mut^s and 13 mut^+). In general, the levels of CPA inhibitory activity were higher in mut^s phenotype than in mut^+ (Fig. 1) and the highest expressing clone (4s) was chosen to analyze the time-dependent expression of PCI.
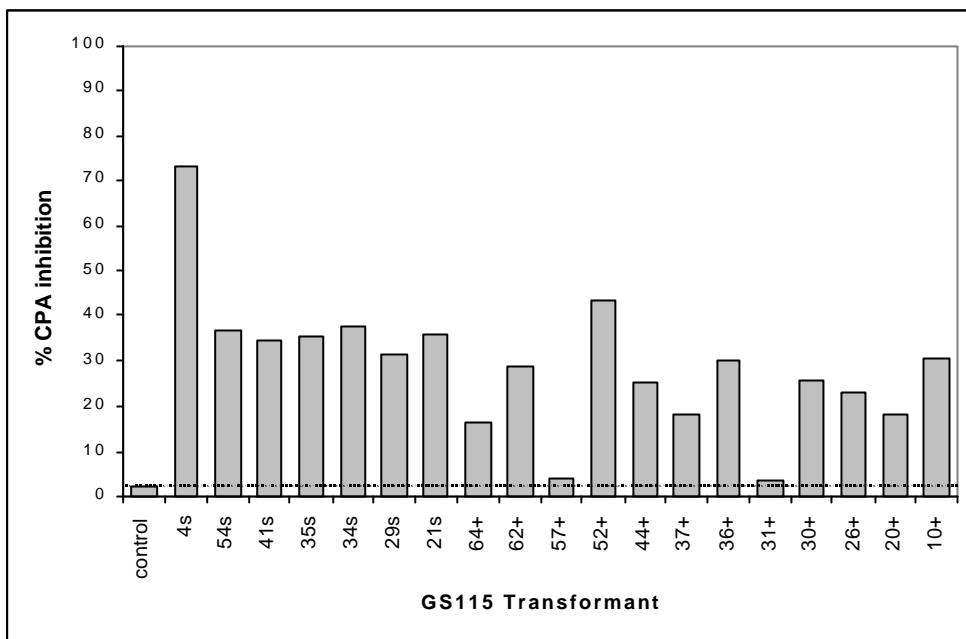
Figure 1. Identification of the *P.pastoris* highest expressing clones by determination of CPA inhibitory activity. CPA activity for each crude supernatant is plotted for GS115 transformants. Each transformant is designated by a number, corresponding to an isolation identifier, and a letter, corresponding to its mut phenotype. The dashed line indicates the level of background activity for the enzymatic assay, which is the activity for a non-induced GS115 transformant. 4S transformant was chosen for further development.

After a 2-day growth phase with BMGY medium the cell pellet was ressuspended in 1/5 volume of BMMY medium and 1% methanol was added every 24 h. The PCI expression on the cell culture was analyzed by CPA inhibitory activity of samples taken in a time-course manner.
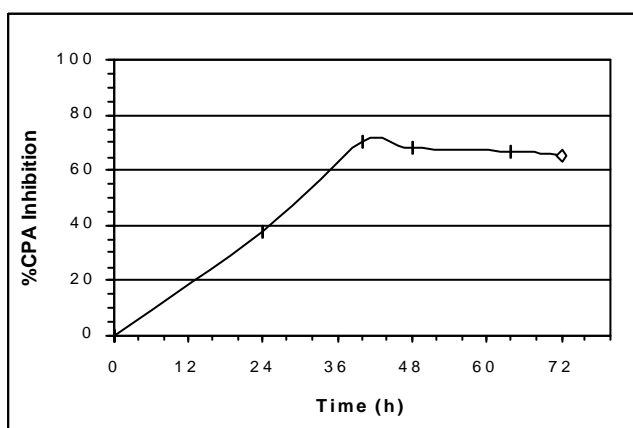

Figure 2. PCI stability in the cell culture. PCI expression of 4S transformant was induced with 1% methanol and was maintained by 1% methanol addition every 24 hours. Samples were taken in a time-course manner and the presence of PCI in the crude supernatant was evaluated by its ability to inhibit CPA.

The results show that the maximum CPA inhibitory activity is reached about 40 h after induction of protein expression and it is maintained at least for the following 32 h, indicating that PCI is not

degraded in the media (Fig. 2). From these data, a 2-day expression phase was chosen to perform the PCI expression in 2-liter flasks.

The most productive clone, 4s, was grown for two days in 400 ml of BMGY in 2-liter flasks, the cell pellets were resuspended in 150 ml of BMMY and 1% methanol was added every 24 h for 48 h. Purification of the PCI from the *P.pastoris* supernatant was accomplished in 3 chromatographic steps (see Material and Methods). The final PCI production was 5.5 mg per liter of initial cell culture, determined by optical density at 280nm -$E_{0.1\%} = 3.0$ (Hass and Ryan, 1981), and the protein was finally analyzed by MALDI-TOF MS. A unique peak with a molecular mass of 4295 Da was identified, which corresponds to mature wt PCI, indicating that the α-factor secretion signal had been well processed and that the mature protein had not undergone any degradation process.

IV.C.2   Expression of pIN3OmpAIII-His-Tag-PCI by E.coli

HistagPCI, a construction coding for 6His+1Arg at the N-terminus of PCI, was expressed from pIN3OmpAIII vector in the *E.coli* MC1061 strain after induction of protein expression with 1 mM IPTG. The 24 h induced supernatant was analyzed by CPA inhibitory activity and the results (data not shown) indicated that the protein was expressed and that at least a significant percentage of the protein was properly folded. MALDI-TOF MS analysis of these samples revealed 3 major molecules present, with molecular weights of 4588, 4451 and 4295 Da, corresponding to His+Arg+PCI, Arg+PCI and PCI, respectively. Apparently, the His-tag-PCI was not correctly processed and it had undergone a degradation process at its N-terminus. Therefore, the molecules that were present in the cell culture did not present the His-tag tail.

His-tag-PCI expression was studied in a time-course manner, taking samples at different times after the cell culture induction, so that the degradation pattern could be determined. The samples, after purification and concentration using a C_{18} resin (see Materials and Methods), were analyzed by MALDI-TOF MS. The three molecules identified in the 24-h induced culture could be detected 15 min after IPTG addition and the same pattern could be observed in the 30-min and 210-min spectra (Fig. 3).
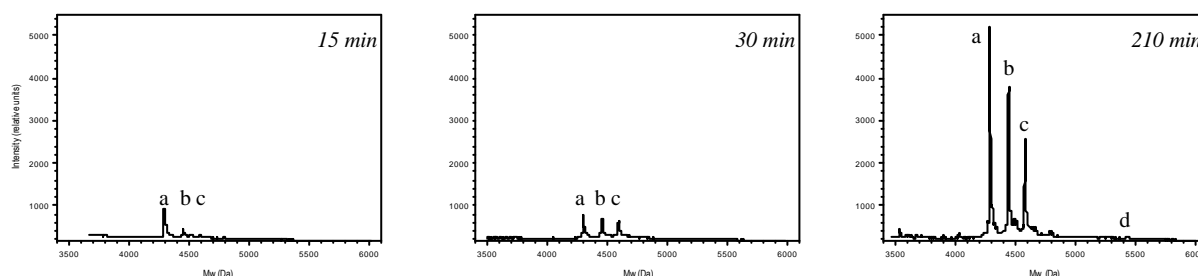

Figure 3. His-tag degradation kinetics. HistagPCI expression was induced with 1mM IPTG, samples were taken in a time-course manner, purified using a C18 resin and analyzed by MALDI-TOF MS. Molecules a, b, c and d display molecular weights of 4295, 4441, 4588 and 5444 Da and correspond to PCI, Arg+PCI, His+Arg+PCI and (His)6+Arg+PCI, respectively.

The periplasmic fraction at 210 min was also studied and it showed the same degradation pattern (data not shown). The complete molecule, containing the His-tag, was only detected by MALDI-TOF MS, 210 minutes after IPTG addition (Fig. 3, 210-min spectra, molecule d), but it gave rise to an extremely weak peak in the mass spectra and it could not be identified by RP-HPLC analysis, indicating that His-tag-PCI was very vulnerable to proteases, leading to three major products of proteolysis and to a hardly noticeable amount of well-processed protein.

IV.C.3   Expression of pBAT4-OmpA-PCI by E.coli

Mature PCI was expressed in *E.coli* BL21 (DE3) cells harboring the plasmid pBAT4-PCI. Analytical expressions were performed in order to evaluate the effect of the carbon source and the inducer on the expression of mature PCI. Two types of carbon sources were tested -glycerol and glucose- and two inducers -IPTG and lactose-. All the cell cultures were induced when the cell density at 550 nm reached 0.7, and after 20 hours were centrifuged, filtered and analyzed by RP-HPLC.

TABLE 1

Effect of the carbon source and the inducer on PCI production

Parameter tested	fold[c]
(glycerol 0.5%) / (glucose 0.5%)	4.3
(glycerol 2%) / (glucose 2%)	3.0
(glycerol 0.5%) / (glycerol 2%)	1.2
(glucose 0.5%) / (glucose 2%)	0.7
(glycerol 0.5%+IPTG[a]) /(glycerol 0.5%+lactose[b])	2.0
(glucose 0.5%+IPTG[a]) / (glucose 0.5%+lactose[b])	8.0

[a] IPTG concentration was 0.2 mM
[b] Lactose concentration was 5 mM
[c] The ratio between the amount of PCI produced in each selected conditions was evaluated by comparison of the peak areas of the corresponding RP-HPLC chromatograms.

A 3 to 4-fold increase in the level of mature PCI was observed when glycerol was used as the carbon source in comparison to glucose, eventhough the cell growth rate was faster using glucose. There were not significant differences when a 0.5% was compared to a 2% glycerol concentration (Table 1). Use of IPTG as an inducer of PCI expression resulted in a clear increase of the mature protein yield, either using glycerol or glucose as the carbon source. According to this, experiments of PCI production in 2-liter flasks were performed using glycerol as the carbon source, at 0.5% concentration and IPTG 0.2 mM as the inducer. After a protein purification using three chromatographic steps (see Material and methods) routine PCI yields were about 15 mg/liter.

IV.D   Discussion

Besides its interest in basic research, PCI presents strong biotechnological applications, in approaches to defense plants from insects, as an adjunct to tPA to improve thrombolytic therapies or as a putative EGF antagonist for cell growth control. The reported yield of PCI produced in shake flasks in our laboratory was very low, about 1-2 mg/liter (Molina et al, 1992). Therefore, it was necessary to develop a new expression system to fulfill the protein requirements of such applied projects and to obtain high enough yields of the new PCI mutant proteins designed in our laboratory, in order to perform folding, kinetic and structural studies. We also took such a challenge as a problem of general interest, because PCI is a small disulfide-rich protein, with a tight conformation -it belogns to the knottin family (Lin and Nussinov, 1995)-, and these are proteins of difficult production by recombinant DNA approaches. To deal with this, we selected three different systems for heterologous expression: one based in external secretion by the yeast *P.pastoris* and two others based in the periplasmic secretion in *E.coli*, using powerful promoters in all cases.

The yeast *P.pastoris* has been broadly used to produce high levels of a wide range of heterologous proteins (Cereghino and Gregg, 2000; Cregg et al, 2000). Many parameters effect the foreign gene expression levels in this yeast, leading to a high clonal variation in expression, such as the gene copy number, the type of expression vector, the methanol utilization phenotype... According to these facts, a wide clone screening was performed to select the strain with the highest protein expression. Out of 20 clones, the transformant 4s was chosen, which gave a final yield of 5.5 mg/liter in the optimal conditions, 3 to 4-fold higher than the previous *E.coli* system used in our laboratory (Molina et al, 1992). Hirudin (Rosenfeld et al, 1996), tick anticoagulant peptide (TAP) (Laroche et al, 1994) and ghilanten (Brankamp et al, 1995), all of them small disulfide-rich proteins, have been expressed and secreted by *P.pastoris* at levels of 1.54 g, 1.7 g and 10 mg/liter respectively. All these three proteins contain multiple intramolecular disulfide bonds, TAP and hirudin contain 3 and ghilanten contains 5 disulfide bonds, and PCI also contains 3 disulfide bonds. The successful expression and folding of these proteins suggests that other disulfide-rich proteins could likewise be produced using this system.

However, PCI expression was not as high as the other reported disulfide-rich proteins, but it should be taken into account that PCI was expressed in 2-liter flasks and the former proteins achieved their highest expressions in bioreactor. For instance, hirudin expression was around 75 mg/liter in shake flasks, and a 20-fold increase was achieved in the scale-up process to a 3-liter bioreactor. Based on the expression of hirudin and other recombinant proteins in *P.pastoris*, a significant improvement in PCI production might be achieved by advancing cultures from shake flask to fermentation conditions (Brierley, 1998; Clare et al, 1991). Besides, the growth conditions for *P.pastoris* are ideal for large-scale productions, because the medium components are inexpensive and free of undefined ingredients that can be sources of pyrogens or toxins.

Protein secretion into the *E.coli* periplasm or the extracellular medium has been largely reported (Pines and Inouye, 1999). PCI has been expressed in our laboratory in *E.coli* using the expression vector

pIN3OmpAIII, which utilizes the strong lipoprotein promoter (*lpp*) (Molina i col, 1992). However, difficulties are associated with the purification of the protein, since three chromatographic steps are required. This long time protocol implies a low protein recovery. In order to achieve a rapid an efficient purification protocol, a $(His)_6$-tag was added to the N-terminal sequence of PCI. This system should allow the PCI purification by a single step using a Ni^{2+} affinity chromatography. Although the protein was expressed, the His-tag was proteolyzed and it led to an accumulation of three major molecules that did not present the His-tag at the N-terminus (PCI, Arg+PCI and His+Arg+PCI). The analysis of this proteolytic process indicated that the degradation pattern in the extracellular medium was not time-dependent and was similar to that observed in the periplasmic fraction. Thus, the results suggested that the degradation took place in the periplasm and not in the extracellular medium, and therefore, the use of protease inhibitors could not be useful. Such a degradation made impossible the use of the Ni^{2+} affinity chromatography to purify the protein and therefore, another expression system in *E.coli* was investigated.

PCI was also expressed in *E.coli* with the vector pBAT4, which derives from the pET vectors and contains the T7 RNA polymerase promoter (Peranen et al, 1996). A signal sequence coding for the OmpA signal peptide was included to obtain a periplasmic expression. The protein expression was performed in the *E.coli* strain BL21 (DE3) and several parameters were optimized for PCI expression. Finally, a 0.2 mM IPTG concentration as inducer and 0.5% glycerol concentration as the carbon source were chosen. PCI expression using glycerol as the carbon source was higher than using glucose; in contrast, the cell growth rate was faster using glucose. This indicates that a rapid cell growth can be a harmful parameter for protein expression. PCI expression in pBAT4 vector is under the control of T7 RNA polymerase promoter. The protein expression under this promoter takes place within few hours after IPTG addition. In an intracellular expression this leads to inclusion bodies formation, which can be afterwards purified (unpublished results), but in an extracellular expression, it probably leads to the overloading of the export machinery at high growth rates, resulting in a reduction of the PCI amount in the extracellular media. The final yield of PCI produced with the pBAT4-OmpA vector in the optimal conditions was about 15 mg/liter in flask.

Although PCI expression in BL21 (DE3) and MC1061 *E.coli* strains was achieved using the signal peptide OmpA, PCI was purified from the extracellular media. The OmpA and the PhoA signal peptides direct expression of the recombinant proteins to the periplasmic space, but the OmpA signal peptide translocates the recombinant proteins to the outer membrane and this facilitates the release of the molecule into the culture medium to a greater extent than does the PhoA signal peptide. Even though the outer membrane of *E.coli* only allows the diffusion of molecules smaller than 600 Da, it has been observed that some proteins find their way to the growth medium of the cells (Somerville et al, 1994; Manosroi et al, 2001). This protein passage from the periplasmic space into the growth medium has been suggested to occur through a non-specific leakage, in a process that is highly protein sequence-specific (Hewinson and Russel, 1993).

This method of production of PCI in a pBAT4 based system in shaken flasks, has increased the yield of PCI up to the quantities required for kinetic, folding and structural studies without the necessity to produce the protein using a bioreactor, which is a much more expensive and time-consuming procedure. The PCI yield of 15 mg/liter in shaken flasks is 10-fold higher than the previous PCI expression system described in *E.coli*, which was about 1-2 mg/liter (Molina et al, 1992), and this allows us to study a wide range of new mutant forms of PCI in our laboratory using shaken flasks to produce them, which implies an important gain of time and money. Although PCI expression in *P.pastoris* was not so high as in *E.coli*, the former system should also be taken into account, since it presents some advantages: The working volume at the beginning of the purification protocol is much reduced compared to that of the *E.coli* expression system (see Materials and Methods); the induction system is much cheaper; and the clonal stability is greater due to the integration of the exogenous gene in its genome; thus, there are not segregation problems as in *E.coli* plasmids. However, a drawback is that the *P.pastoris* expression system takes 4 days to accomplish the whole growing and expressing process, whilst only 2 days are required in the *E.coli* system.

PCI production in both systems could be further improved by scaling-up the expression in a controlled bioreactor. The advantages of bioreactor expression in *P.pastoris* have been already discussed. For *E.coli*, PCI production in a 2-liter bioreactor has been previously reported using a pIN3OmpAIII vector (Marino-Buslje et al, 1994). In that case, the scale-up process from shaken flasks to bioreactor allowed a 200-fold improvement. In fact, the scale-up process is currently being tested in our laboratory, since a similar increase in our PCI production would lead to a final production of 2-3 g/liter .

In summary, PCI can be efficiently expressed by and purified from *P.pastoris* and *E.coli*. PCI produced by both expression systems is fully active, since inhibits CPA activity with the expected kinetic parameters, showing a standard RP-HPLC behaviour and the molecular weight identified by MALDI-TOF MS corresponds to that of mature PCI. Only His-tag-PCI shows degradation problems that make impossible its purification by a single-step metal affinity chromatography. Expression of PCI in *E.coli* BL21 (DE3) using the pBAT4 vector produces 15 mg/liter of protein in shaken flasks, 10-fold higher than the previously described system.

IV.E   References

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