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Recombinant expression of disulfide-rich proteins: carboxypeptidase inhibitors as model proteins

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Background

A large number of physiologically relevant proteins comprise disulfide bonds: protease inhibitors, proteases, nucleases, growth factors, venom neurotoxins, and others. The recombinant expression of these proteins usually represents an important challenge due to the presence of disulfide bonds, which may affect protein solubility inside the cell and lead to misfolding and subsequent aggregation.

In the last years, specific inhibitors of carboxypeptidases of the M14A and M14B subfamilies have emerged as potential drugs able to protect plants against insect attack and to improve blood clot fibrinolysis [1]. However, the small size and high number of disulfide bonds present in these molecules strongly influence their recombinant production and further biochemical characterization. Here, we have tested different systems for the successful expression of four carboxypeptidase inhibitors from the potato plant (PCI; 39 residues and 3 S-S), the intestinal parasite *Ascaris suum* (ACI; 65 residues and 4 S-S), the medical leech *Hirudo medicinalis* (LCI; 66 residues and 4 S-S), and the soft tick *Rhipicephalus bursa* (TCI; 75 residues and 6 S-S)[2].

Results

The recombinant expression of PCI in the methylotrophic yeast *Pichia pastoris*, using the pPCI9 vector (Invitrogen), resulted in a soluble and properly folded protein that was secreted into the medium to a final yield of 5 mg/liter. The utilization of the *E. coli* protease-deficient strain BL21(DE3) and the vector pBAT4-OmpA [3] routinely yielded 15 mg/liter of recombinant protein, 10-fold higher than a previously described *E. coli* system (pIN3OmpAIII vector in *E. coli* strain MC1061) [4]. However, the scale-up from flask culture to fed-batch fermentor did not improve the previous system (70 *versus* 200 mg/liter) [5]. Importantly, the PCI expressed in *E. coli* was completely folded by addition of redox agents to the culture supernatant (2 mM Cys-Cys/4 mM Cys, pH 8.5). Intracellular expression of PCI as a thioredoxin fusion protein in *E. coli* ADA494 using the pET-32b vector led to extremely low yields of soluble protein.

Recombinant LCI was cloned and expressed in periplasm using *E. coli* BL21(DE3) cells and the pBAT4-OmpA vector. Approximately 5 mg/liter of recombinant LCI were produced in shaker flasks, a moderate improvement compared with the previously reported expression systems in *E.coli* using MC1061 and ADA494 cells with the pIN3OmpAIII and pET-32b vectors, respectively, both yielding 3 mg/liter [6]. The addition of redox agents also rendered native folded protein. Recombinant TCI was expressed in *E. coli* BL21(DE3) cells using the pBAT4-OmpA vector, with a yield of 1 mg/liter. Intracellular expression attempts did not render recombinant TCI. Similar to PCI and LCI, the presence of redox agents was necessary to achieve a folded inhibitor. Scale-up studies for

LCI and TCI were performed in a fed-batch fermentor using the conditions optimized for PCI. However, low yields of recombinant protein were obtained for both molecules (<5 mg/liter).

Currently, we are working on the recombinant expression of ACI. This protein has been cloned in the pPICZα vector (Invitrogen) for expression in *Pichia pastoris* and in the pBAT4-OmpA and pET-32b vectors for expression in *E. coli*. Initial results point out yeast as the most useful system for the expression of recombinant ACI. Intracellular expression systems are also being tested in *E. coli*.

Conclusion

The use of BL21(DE3) cells and pBAT4-OmpA vector in combination with redox agents results in soluble and properly folded protein for all tested inhibitors, indicating that the periplasmic expression is a good solution to cope with the problems related to disulfide bond formation. However, the scale-up of this system does not render high protein yields. On the other hand, intracellular expression of these proteins usually leads to misfolding, aggregation and inclusion bodies formation.

For all proteins, the extracellular expression in the yeast *P. pastoris* seems to be an adequate alternative to overcome the folding problems. Finally, the overall results indicate that the higher the number of disulfide bonds, the lower the protein expression yield.

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