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ORIGINAL PAPER

Expression of metallocarboxypeptidase inhibitors in *Escherichia coli*: effect of cysteine content and protein size in the secretory production of disulfide-bridged proteins

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Abstract Metallocarboxypeptidase inhibitors are proteins with possible applications in biomedicine given their properties as anticoagulant and antitumoral factors. They are small, eukaryotic polypeptides comprising several disulfide bridges, which makes them hard to express in inexpensive bacterial hosts. In this work, three of them were produced in high-cell-density cultures of *Escherichia coli*: PCI (39 residues and three bridges), LCI (66 residues and four bridges) and TCI (75 residues and six bridges). The genes coding for the mentioned inhibitors were cloned in an arabinose-inducible plasmid fused to the signal peptide of DsbA in order to have them secreted and grant the formation of the bridges. The trigger-factor defective strain KTD101 was used as the expression host. The resulting recombinant strains were cultured in fed-batch mode employing minimal media and an exponential feed profile, keeping the specific growth rate at $\mu = 0.1\ h^{-1}$ by limitation of the fed carbon source (glycerol). Between 380 and 540 mg l^{-1} of active inhibitors were obtained in both the periplasmic extracts and extracellular media of the cultures. Later on, excretion was enhanced using a cell permeabilization treatment, allowing the recovery of over 80% of the products from the extracellular fraction. Protein yields were found to be inversely proportional to cysteine content of the inhibitor, whereas protein excretion rates were inversely proportional to the protein size. Overall, these results offer insight into the secretory production of active disulfide-bridged proteins in high-cell-density cultures of *E. coli*.

Keywords High-cell-density cultivation · Recombinant secretion · Disulfide bridges

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Introduction

Metallocarboxypeptidases (MCPs) are exopeptidases of known ubiquity in the animal kingdom. In the past decade, new MCPs have been identified in mammalian tissues and fluids, and their role in a range of physiological processes has been established. Hence, it comes as no surprise for them to be new targets in biomedicine [8]. Conversely, metallocarboxypeptidase inhibitors (MCPIs) constitute promising molecules for therapeutic purposes, as regulators in pathological processes where MCPs might be involved. There are only a handful of inhibitors of peptides known to specifically bind to MCPs [2], which have been found in potatoes, tomatoes, roundworms, leeches, ticks, and rat brain. All of them are small proteins comprising disulfide bridges, which makes their recombinant expression more difficult.

An interesting alternative for the production of disulfide-bonded proteins in *Escherichia coli* is to target them to the periplasmic space, where a battery of enzymes and an oxidizing environment promote the formation of disulfide bridges [4]. In some cases, recombinant products leak to the extracellular milieu, a phenomenon much less understood than export through the inner membrane. However,

achieving quantitative yields of secreted/excreted recombinant proteins in *E. coli* can be complicated, mainly because the performance of its translocation apparatus is affected by a number of issues, including the nature of the exported protein itself. Successful protein secretion can be affected by genetic factors [13, 14] such as the promoter strength, the nature of the signal sequence or plasmid copy number, and cultivation variables like temperature, culture media, and growth kinetics [11, 12, 21]. Still, secretory expression in *E. coli* is attractive from a downstream processing standpoint, since no cell-disruption steps are needed, and contamination with other proteins is reduced both in the periplasm and the culture broth [9, 23].

For characterization purposes, recombinant MCPIs have been expressed in the periplasm and culture media of *E. coli* at the shake-flask level [1, 15, 19] but scale-up for expression at bioreactor level proved difficult or not possible. The potato carboxypeptidase inhibitor (PCI) has been produced in high-cell-density cultures using the expression/secretion vector pIMAM3, derived from plasmid pIN-III-ompA3, which fused the PCI gene to the ompA signal sequence and operated under the transcriptional control of a hybrid *lpp* promoter [15]. Recently, we transformed this vector in the strain BW25113 and cultured the recombinant strain in minimal medium applying an optimized fed-batch protocol [16]. Around 700 mg l^{-1} of the inhibitor were obtained under these conditions. Production scale-up of other larger inhibitors like the one from leeches and that from ticks (LCI and TCI, respectively) was not possible with pIN-III-ompA3 due to leaky expression compromising cell viability (data not shown). To overcome this, a new expression vector named pLCB was constructed from pBAD33 [10]. This plasmid contains the arabinose-inducible and tightly regulated PBAD promoter and a relatively low copy number to avoid leaky expression. With this system, over 450 mg l^{-1} LCI was obtained in high-cell-density cultures using complex media [16]. Interestingly, for both cases, distribution of the product along the periplasmic space and culture media depended highly on cultivation conditions, suggesting that process strategies could be manipulated in order to increase protein secretion and excretion.

With the explained record in mind, this work focuses on the development of a fed-batch process to produce three of these inhibitors, namely PCI, LCI, and TCI (Table 1), using a robust fermentation process. Our aims were not only to express these inhibitors but to study the production of disulfide-bridged proteins and investigate the effect of the number of disulfide-bridges and protein size in their expression, secretion, and excretion dynamics. Given the good results obtained for the expression of LCI, it was decided to clone the PCI and TCI genes into the pLCB vector. The recombinant strains were cultured in fed-batch using minimal synthetic media and an exponential feed profile. For all three inhibitors, the concentration profiles in the cytosol, periplasmic space, and culture media were analyzed in order to identify and overcome the bottlenecks in the secretory production of these proteins.

Materials and methods

Reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated.

Strains and plasmid

E. coli strain KTD101 (Δ(*araD-araB*)*567 ΔlacZ4787* (::*rrnB-3*) *lacI*^{p}*-4000*(*lacI*^{Q}) *λ*$^{-}$ *rph-1* D(*rhaD-rhaB*)*568, hsdR514* Δ*tig100*) was used as host. The pBAD33-derived plasmid pLCB (Fig. 1) [16] was used as the expression vector.

Cloning of PCI and TCI into the KTD101/pLCB system

Plasmid pLCB, a pBAD33 derivative, had already been constructed for the expression of LCI in high-cell-density cultures of *E. coli* [16]. In the latter work, different leader peptides were tested in order to maximize the amounts of secreted LCI. The tested signal sequences were those of the native *E. coli* proteins ompA, dsbA, and malE, which are all secreted from the bacterial cytosol.

In order to eliminate rare codons that could result in suboptimal transcription rates, gene sequences encoding for PCI and TCI were optimized and chemically synthesized (Geneart, Regensburg, Germany). The PCI and TCI genes were then cloned into the BamHI/HindIII sites of the pLCB version containing the desired signal sequence to build pLCB-ompA-PCI, pLCB-dsbA-PCI, pLCB-malE-PCI, pLCB-ompA-TCI, pLCB-dsbA-TCI, and pLCB-malE-TCI.

Table 1 Metallocarboxypeptidase inhibitors produced in this work

Inhibitor	Natural source	Residues (−)	Disulfide bridges (−)	Cysteine content (%)
PCI	Potatoes (*Solanum tuberosum*)	39	3	0.13
LCI	Leeches (*Hirudo medicinalis*)	66	4	0.11
TCI	Ticks (*Rhipicephalus bursa*)	75	6	0.15

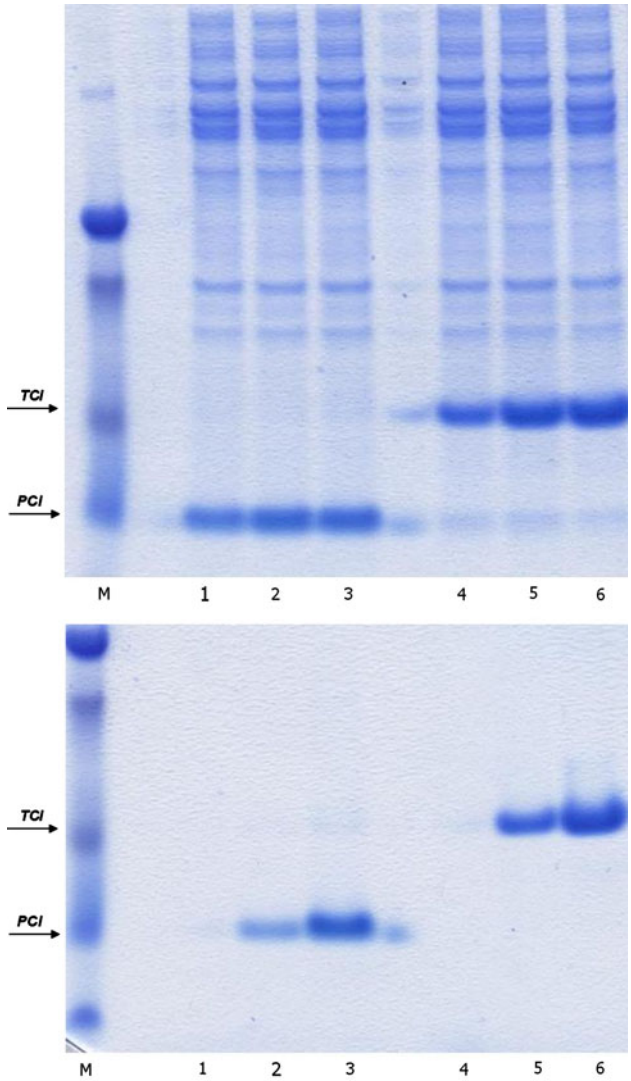

Fig. 1 Steady-state level and distribution of PCI and TCI. Soluble whole-cell extracts (*top*) and purified supernatants (*bottom*) were separated by SDS–PAGE and stained by colloidal Coomassie Blue. *M* molecular weight marker; *1* pLCB-ompA-PCI; *2* pLCB-malE-PCI; *3* pLCB-dsbA-PCI; *4* pLCB-ompA-TCI; *5* pLCB-malE-TCI and *6* pLCB-dsbA-TCI

These six expression vectors were transformed in the strain KTD101, which is defective for the expression of the ribosomal protein trigger factor (TF). In a related study performed with LCI as a model protein [17], it was described how deletion of TF resulted in higher levels of secreted proteins via the SRP pathway.

Shake-flask cultivation conditions

For shake-flask experiments, either rich Luria–Bertani (LB) or minimal (MDE) media supplemented with 30 μg/ml chloramphenicol were used. The media composition is detailed elsewhere [18].

Seed cultures were prepared in 15-ml culture tubes by inoculating 5 ml of LB with a single colony from a fresh transformation plate. Tubes were incubated overnight at 37°C with vigorous agitation. Shake-flask cultures were done in 500-ml baffled-shake flasks by inoculating 100 ml of fresh MDE media with 1 ml of seed culture; these cultures were then incubated under the same conditions as the seed cultures. Induction was performed by adding L-arabinose from a 20% sterile stock.

Bioreactor cultivation conditions

Fed-batch cultivation experiments were performed using a 2-l jar and a standard Biostat B® digital control unit. A fed-batch cultivation protocol using minimal media was used to grow the strains to high cell densities. An initial discontinuous phase using glucose as the carbon source was used to grow biomass overnight. Once the glucose was depleted, addition of feedstock containing glycerol was started according to an exponential feed profile, which allowed fixing the specific growth rate at $\mu = 0.1\ h^{-1}$ by limiting the carbon source. This cultivation protocol and media used are thoroughly described elsewhere [18].

Biomass analyses

Cell growth was monitored by turbidity measurements at 600 nm (OD_{600}) using a spectrophotometer (UNICAM 8625). Turbidity was correlated to dry cell weight (DCW) through a calibration curve constructed by standard methods [22]. Estimation of plasmid-bearing cells was done by comparing the number of CFUs on plain LB-agar plates and on LB-agar plates supplemented with 30 μg ml^{-1} chloramphenicol, after plating properly diluted samples of culture broth.

Cell fractionation and culture supernatant preparation

Fractionation of culture samples into spheroplasts, periplasmic extracts, and supernatants was accomplished as detailed in [3]. Soluble cytosolic proteins were released by sonication of spheroplasts using a Vibracell® model VC50 (Sonics & Materials). Inhibitors from extracellular fractions were purified using atmospheric pressure reverse-phase chromatography columns, SepPak C18 (1 g) (Waters): Clear supernatants (40 ml) were loaded on the column, previously equilibrated with acetonitrile and rinsed with ultrapure water. Columns were washed with 4 ml of 10% acetonitrile and 4 ml of ultrapure water previous to elution with 4 ml of 30% isopropanol. When needed, supernatants were concentrated using Amicon or Minicon centrifugal filter devices (Millipore).

Protein electrophoresis

Proteins were separated and visualized on 12% Bis–Tris electrophoresis gels from the Novex system (Invitrogen) using MES-SDS as running buffer. Gels were stained with colloidal Coomassie Blue.

Reverse-phase HPLC quantification

Reverse-phase liquid chromatography was employed to separate and quantify active inhibitors (PCI, LCI, and TCI) in periplasmic and supernatant fractions. An Ultimate 300 HPLC system (Dionex) and a Protein & Peptide C_4 cartridge (Vydak) were employed, with H_2O/acetonitrile as the mobile phase. Elution of the inhibitors was done over a gradient of acetonitrile (pH = 1.00) from 20 to 80%. Using standards of purified protein, a calibration curve was constructed to estimate the concentration of active inhibitor in the injected samples.

Results

Shake-flask experiments

First, the new PCI- and TCI-expressing strains were screened to determine which signal peptide gave out the best yields of secreted inhibitors, in accordance to what had been done in [16] for LCI. The six generated recombinant strains (see "Materials and methods") were grown in 100 ml of LB broth supplemented with antibiotic and induced with 0.2% arabinose in mid-exponential phase (OD = 0.6). After 5 h of induction, the cells were harvested and fractioned, and supernatants were purified. Analysis of the supernatants and whole-cell extracts (Fig. 1) revealed that the dsbA signal sequence promoted higher levels of protein secretion for both PCI and TCI, suggesting that cotranslational export via the SRP pathway constitutes a viable alternative for the secretion of recombinant products [20] and is more adequate for this family of proteins. Interestingly, no significant amounts of immature pre-secretory protein were detected in whole-cell extracts, indicating that quantitatively all recombinant product had been exported.

Small-scale experiments in MDE broth were performed as a first approach to expression of the inhibitors in minimal media employing the strains KTD101 (pLCB-dsbA-PCI) and KTD101 (pLCB-dsbA-LCI), and also KTD101 (pLCB-dsbA-TCI). The three strains coding for the different inhibitors, as well as control cells carrying the empty pLCB plasmid, were cultured in 500-ml baffled shake flasks. Once the turbidity reached OD = 0.6, arabinose was added to a final concentration of 0.2% and the cultures were allowed to grow for 5 additional hours while monitoring their growth. As expected, induction produced a decrease on the specific growth velocity; and the time that cells could grow before suffering a growth arrest slightly varied from one strain to the other (Fig. 2). After induction, culture supernatants were purified and cells were fractioned. The amount of active inhibitors in periplasmic extracts and cultures supernatants were quantified by RP-HPLC (Table 2), and it became clear that product distribution was dependant on the identity of the inhibitor.

Production of inhibitors in fed-batch cultures

Fermentor cultures were performed according to the fed-batch protocol described in the "Materials and methods" section and used successfully in other works of our research group [6, 7, 18]. Over 66 g DCW $\cdot$ l^{-1} ($OD_{600nm} \approx 200$) was attained upon cultivation of the strain KTD101 bearing the empty pLCB plasmid. Specific growth rate during the fed stage was set at $\mu = 0.10\ h^{-1}$. The end of the process

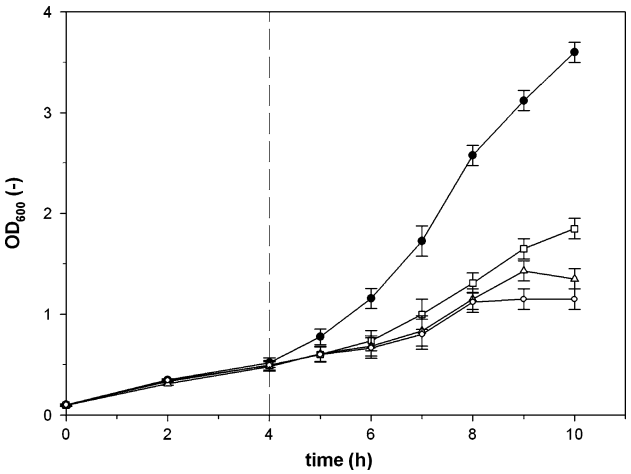

Fig. 2 Growth curves of the recombinant strains in shake-flask cultures. KTD101 cells carrying the empty pLCB (*filled circle*), pLCB-dsbA-PCI (*open square*), pLCB-dsbA-LCI (*open triangle*), and pLCB-dsbA-TCI (*open circle*), were grown at 37°C in MDE medium supplemented with 30 μg ml^{-1} chloramphenicol. The induction moment is indicated with a *dashed line*

Table 2 Expression of PCI, LCI, and TCI in shake flasks using MDE medium

Inhibitor	Periplasm (μg ml^{-1} broth)	Culture media (μg ml^{-1} broth)	Total (μg ml^{-1} broth)
PCI	7 ± 2	15 ± 3	22 ± 5
LCI	12 ± 3	13 ± 2	25 ± 5
TCI	15 ± 2	5 ± 1	20 ± 3

Final amounts of recombinant inhibitor per unit of culture volume, as assessed by RP-HPLC

was marked by the depletion of dissolved oxygen, even when pure oxygen was used as the inlet gas.

Once the efficacy of the fed-batch protocol was validated, we proceeded to carry out production cultures for each inhibitor, setting the specific growth velocity at $\mu = 0.10\ h^{-1}$. Protein expression was accomplished by the addition of arabinose to 1.0% final concentration. The choice of the induction moment for fed-batch cultures was done bearing in mind the patterns observed in the shake-flask cultures: from the growth curves in Fig. 2, the number of duplications post-induction that each strain could endure was estimated (1.5 duplications for the PCI-producing strain, 1.1 LCI, and 0.9 for TCI). Accordingly, cells were induced so that the maximum biomass concentration attained in the non-induced culture could be reached [7, 22].

For all three fermentations, the specific growth rate was kept at $\mu = 0.10 \pm 0.06\ h^{-1}$, attaining a final biomass concentration very similar to that of the non-induced culture. Concentrations of product in culture supernatants and periplasmic extracts (per liter of fermentation broth) and growth curves are presented in Fig. 3, whereas Fig. 4 presents SDS–PAGE analysis of final periplasmic and extracellular fractions. It is noteworthy that no pre-secretory inhibitors were detected in the cytoplasm of sample cells, i.e., most of the expressed protein was correctly secreted. Plasmid-bearing cells never dropped under 93% in any of the cases.

Accumulation on the periplasm occurs at the initial stage of the induction phase, even though excretion to the culture media also occurs. However, after a certain concentration threshold, extracellular release from the periplasm is enhanced, which reinforces the hypothesis that excretion of recombinant products is favored by an increase in the osmotic pressure within the periplasmic space [9, 21]. Despite this tendency, the amounts of excreted product varied greatly according to the inhibitor, as did the final concentrations of protein in the periplasmic extracts. These disparities in the final distribution of the inhibitors seem to point out that the size of the protein greatly influences the extent to which it can be excreted (which will be further discussed later).

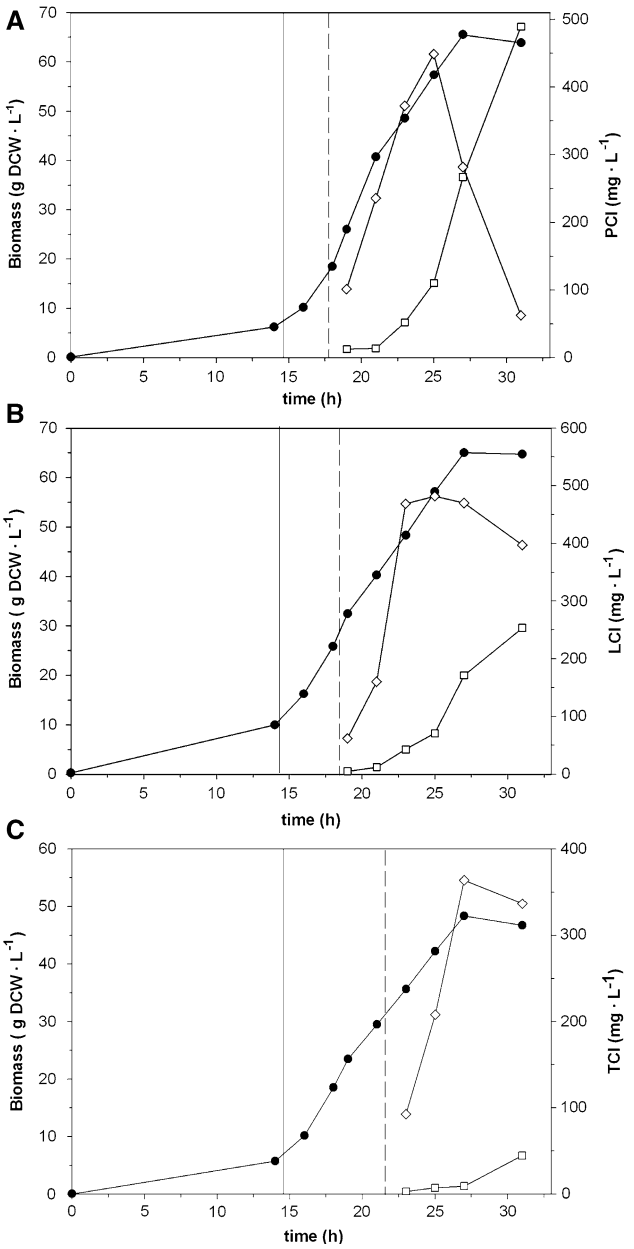

Fig. 3 Production of PCI, LCI, and TCI in a 2-l fermentor. Time-course of induced fed-batch cultures of **a** KTD101(pLCB-dsbA-PCI), **b** KTD101(pLCB-dsbA-LCI), and **c** KTD101(pLCB-dsbA-TCI). Biomass concentration (*filled circle*), and inhibitor production in the periplasmic extracts (*open lozenge*) and culture media (*open square*) are plotted. The start of the fed-batch phase is marked with a *continuous line* and the induction moment is indicated with a *dashed line*

Analysis of protein expression, secretion, and excretion rates

As observed in a related work in the production of PCI with a pIN-III-ompA-derived plasmid [18], the protein-distribution pattern divides the induced phase into two differentiated regions, a first one where the recombinant products is mainly accumulated in the cell, and another where protein production occurs at a lower rate and excretion is favored; these are named production dominant (PD) zone and secretion dominant (SD) zone, respectively. Transition from the first to the second is shown by the decrease of the protein content in the periplasmic extracts.

Protein production rates (q_P, in mg total inhibitor · mg^{-1} DCW h^{-1}) and protein excretion rates (q_E, in mg excreted inhibitor · mg^{-1} DCW h^{-1}) were calculated for each sample time point of the fed-batches. For every inhibitor

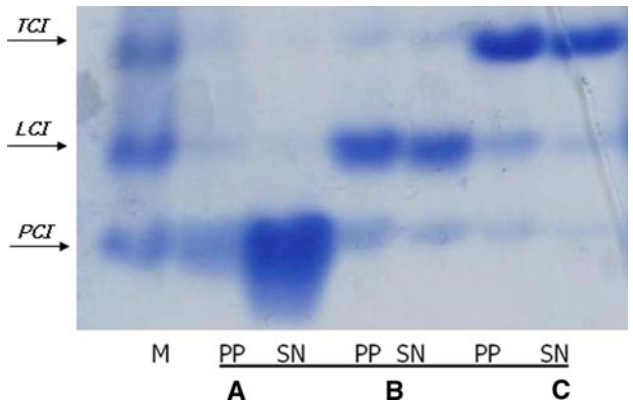

Fig. 4 Final level and distribution of PCI, LCI, and TCI. Final samples of periplasmic extracts (PP) and culture supernatants (SN) drawn from fed-batch cultivation of *a* KTD101(pLCB-dsbA-PCI), *b* KTD101(pLCB-dsbA-LCI), and *c* KTD101(pLCB-dsbA-TCI)

separately, q_P and q_E values were almost constant within each zone, so we calculated mean production and excretion rates, Q_P and Q_E respectively, in the PD and SD zones, with small standard deviations associated. As expected, Q_P values were higher in the PD zone, dropping down in the SD zone; whereas Q_E followed the opposite trend.

If values of Q_P for each process are compared, however, it is clear that the velocity at which each of the inhibitors are produced is influenced by the relative amount of disulfide bridges in its structure, i.e., the ratio of cysteines to total residues in its amino acid sequence (Fig. 5a)—an observation in line with previous studies on the expression of other proteins containing disulfide bridges [5]. Lower yields are obtained for TCI, the one with higher cysteine content (i.e., more disulfide bridges per residue). Conversely, yields of LCI were the highest. Since the process of disulfide bond formation is energy- and time-consuming, we speculate that a higher cysteine content decreases the rate at what the active protein is synthesized.

On the other hand, protein-excretion rates (Q_E) are inversely proportional to the number of residues of the expressed inhibitor (Fig. 5b), suggesting that extracellular product accumulation is related to protein size. In another work [11], it was concluded that protein size did not affect periplasmic translocation via the Sec pathway for peptides ranging between 17 and 42 kDa, which a priori costrasts with our findings. However, Q_E takes into account passage out of the periplasm trough the outer membrane, which is a rather unspecific transport (i.e., not governed by internal cell machinery) compared to translocation from the cytosol to the periplasmic space. This seems to point out that excretion through the outer bacterial membrane is favored for smaller proteins, and gives a hint about the mechanistics of excretion (i.e., it can be somehow seen as passive diffusion through a membrane).

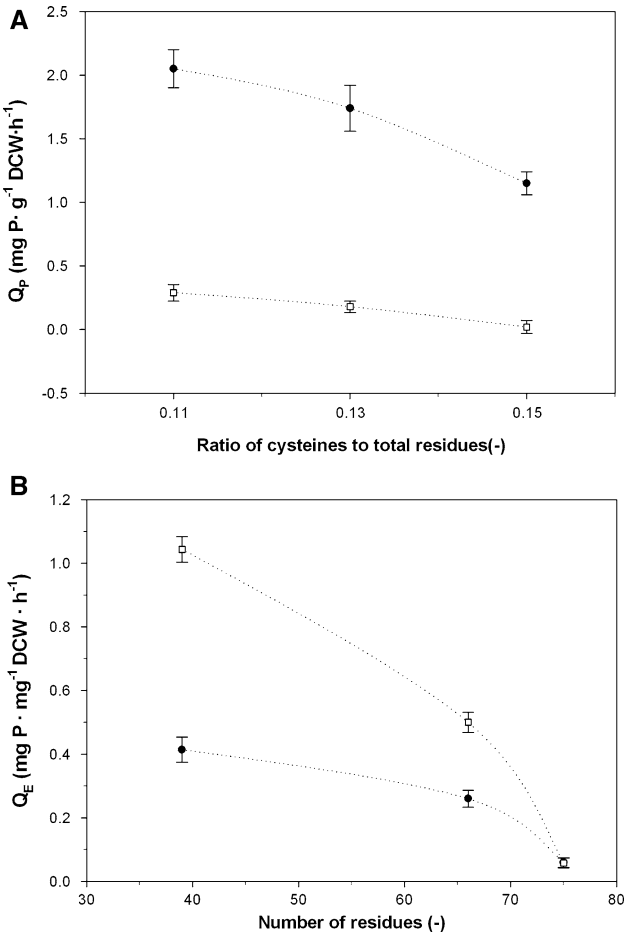

Fig. 5 Inhibitor production and excretion rates' dependence on their identity, as calculated from fed-batch experiments. **a** Mean specific protein production rates (Q_P) in the production dominant (*open square*) and excretion dominant (*filled circle*) zones versus cysteine content. **b** Mean specific protein excretion rates (Q_P) in the production dominant (*open square*) and excretion dominant (*filled circle*) zones correlated to protein size

Enhancement of protein excretion by cell permeabilization

At the end of the production of PCI, more than 90% of this inhibitor could be recovered in the extracellular fractions. However, in the case of LCI and TCI, a significant proportion of the recombinant product was localized in the periplasmic extracts. In order to increase the amounts of secreted protein in the production of LCI and TCI, we implemented a cell permeabilization treatment that had been previously used in a related study [18]. Triton X-100 and glycine, at 0.5 and 2%, respectively, were added to a sample of culture broth at the end of the culture. After 2 h, the distribution of protein between treated and control samples was compared. As seen in Fig. 6, by means of this in situ permeabilization, most of the product is released into the culture broth: 82% for LCI and 81% for TCI. This

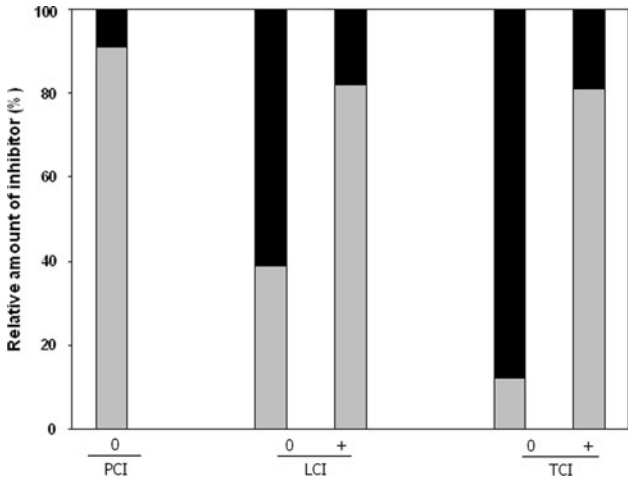

Fig. 6 Effect of cell permeabilization with Triton X-100 + glycine over final LCI and TCI distribution. Distribution of inhibitors in periplasmic extracts (*black*) and culture supernatants (*gray*) at the end of fed-batch cultivation with (+) and without (0) cell permeabilization treatment

is interesting from a downstream processing standpoint since the osmotic shock step to recover the inhibitors from the periplasmic space could be skipped and the product will be present mainly in only one fraction of the culture.

Conclusions

In the present study, coupling of expression system design and fermentation engineering resulted in the successful production metallocarboxypeptidase inhibitors over 300 mg l^{-1}. The designed protocol allowed obtaining significant quantities of fully active proteins, including TCI, which had not been possible before at the bioreactor level due to its complex structure comprising six disulfide bridges. The process presents several advantages: (1) a simple minimal media was used to obtain high levels of biomass; (2) a robust expression system, with high plasmid-retention rates even in high-cell-density cultivation; (3) inexpensive induction by the sugar arabinose, also with reduced toxicity; and (4) full secretion of the recombinant product, with no accumulation of immature form in the cytoplasm. The data presented here shows how global protein production rates are linked to the cysteine content of the protein, and how their secretion depends directly on their size. In order to boost protein excretion and recover most of the product from the culture broth, a cell-permeabilization treatment was applied, resulting in more than 80% of the target products in the extracellular fraction. Overall, our results present interesting strategies for the production of disulfide-bridged proteins and, more generally, of secreted proteins in *E. coli*.

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References

1. Arolas JL, Lorenzo J, Rovira A, Castellà J, Aviles FX, Sommerhoff CP (2005) A carboxypeptidase inhibitor from the tick *Rhipicephalus bursa*: isolation, cDNA cloning, recombinant expression, and characterization. J Biol Chem 280(5):3441–3448
2. Arolas JL, Vendrell J, Aviles FX, Fricker LD (2007) Metallocarboxypeptidases: emerging drug targets in biomedicine. Curr Pharm Des 13(4):349–366
3. Betton JM, Boscus D, Missiakas D, Raina S, Hofnung M (1996) Probing the structural role of an alpha beta loop of maltose-binding protein by mutagenesis: heat-shock induction by loop variants of the maltose-binding protein that form periplasmic inclusion bodies. J Mol Biol 262(2):140–150
4. Choi JH, Lee SY (2004) Secretory and extracellular production of recombinant proteins using *Escherichia coli*. Appl Microbiol Biotechnol 64:625–635
5. de Marco A (2009) Strategies for successful recombinant expression of disulfide bond-dependent proteins in *Escherichia coli*. Microb Cell Fact 8:26
6. Durany O, Bassett P, Weiss AM, Cranenburgh RM, Ferrer P, López-Santín J, de Mas C, Hanak JA (2005) Production of fuculose-1-phosphate aldolase using operator-repressor titration for plasmid maintenance in high cell density *Escherichia coli* fermentations. Biotechnol Bioeng 91(4):460–467
7. Durany O, de Mas C, López-Santín J (2004) Fed-batch production of recombinant fuculose-1-phosphate aldolase in *E. coli*. Process Biochem 40:707–716
8. Fernández D, Pallarès I, Vendrell J, Avilés FX (2010) Progress in metallocarboxypeptidases and their small molecular weight inhibitors. Biochimie (in press). doi:10.1016/j.biochi.2010.05.002
9. Georgiou G, Segatori L (2005) Preparative expression of secreted proteins in bacteria: status report and future prospects. Curr Opin Biotechnol 16(5):538–545
10. Guzman LM, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol 177(14): 4121–4130
11. Mergulhão FJ, Monteiro GA (2007) Analysis of factors affecting the periplasmic production of recombinant proteins in *Escherichia coli*. J Microbiol Biotechnol 17(8):1236–1241
12. Mergulhão FJ, Monteiro GA, Larsson G, Bostrom M, Farewell A, Nyström T, Cabral JM, Taipa MA (2003) Evaluation of inducible promoters on the secretion of a ZZ proinsulin fusion protein in *Escherichia coli*. Biotechnol Appl Biochem 38:87–93
13. Mergulhão F, Monteiro G, Larsson G, Sanden A, Farewell A, Nystrom T, Cabral JM, Taipa M (2003) Medium and copy number effects on the secretion of human proinsulin in *Escherichia coli* using the universal stress promoters uspA and uspB. Appl Microbiol Biotechnol 61:495–501
14. Mergulhão FJ, Summers DK, Monteiro GA (2005) Recombinant protein secretion in *Escherichia coli*. Biotechnol Adv 23(3): 177–202
15. Molina MA, Aviles FX, Querol E (1992) Expression of a synthetic gene encoding potato carboxypeptidase inhibitor using a bacterial secretion vector. Gene 116:129–138

16. Puertas JM, Betton JM (2009) Engineering an efficient secretion of leech carboxypeptidase inhibitor in *Escherichia coli*. Microb Cell Fact 29:57
17. Puertas JM, Nannenga B, Dornfeld KT, Betton JM, Baneyx F (2010) Enhancing the secretory yields of leech carboxypeptidase inhibitor in *Escherichia coli*: influence of trigger factor and signal recognition particle. Protein Exp Purif 74(1):122–128
18. Puertas JM, Ruiz J, Rodriguez de la Vega M, Lorenzo J, Caminal G, González G (2010) Influence of specific growth rate over the secretory expression of recombinant potato carboxypeptidase inhibitor in fed-batch cultures of *Escherichia coli*. Proc Bio 45:1334–1341
19. Reverter D, Vendrell J, Canals F, Horstmann J, Aviles FX, Fritz H, Sommerhoff CP (1998) A carboxypeptidase inhibitor from the medical leech *Hirudo medicinalis*. Isolation, sequence analysis, cDNA cloning, recombinant expression, and characterization. J Biol Chem 273(49):32927–32933
20. Schierle CF, Berkmen M, Huber D, Kumamoto C, Boyd D, Beckwith J (2003) The DsbA signal sequence directs efficient, cotranslational export of passenger proteins to the *Escherichia coli* periplasm via the signal recognition particle pathway. J Bacteriol 185(19):5706–5713
21. Shokri A, Sandén AM, Larsson G (2003) Cell and process design for targeting of recombinant protein into the culture medium of *Escherichia coli*. Appl Microbiol Biotechnol 60(6):654–664
22. Vidal L, Ferrer P, Alvaro G, Benaiges MD, Caminal G (2005) Influence of induction and operation mode on recombinant rhamnulose 1-phosphate aldolase production by *Escherichia coli* using the T5 promoter. J Biotechnol 118(1):75–87
23. Wan EW, Baneyx F (1998) TolAIII co-overexpression facilitates the recovery of periplasmic recombinant proteins into the growth medium of *Escherichia coli*. Protein Expres Purif 14:13–22