GENETIC AND PROCESS ENGINEERING FOR THE RECOMBINANT PRODUCTION OF PROTEIN THERAPEUTICS FOR THE TREATMENT OF CNS DISORDERS

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Ad Elettra Yvonne,
per la sua tenacia

Index

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Ingegnerizzazione genetica e di processo per la produzione ricombinante di proteine terapeutiche per il trattamento di disordini del SNC

Riassunto

Introduzione

I disordini neurologici costituiscono una delle cause principali di disabilità al mondo con enormi conseguenze sulla qualità media della vita delle persone affette e di quella delle persone loro vicine (Feigin *et al.*, 2017). In particolare, con l'incremento delle aspettative di vita, patologie quali il morbo di Alzheimer e quelle associate a varie forme di demenza sono da anni rientrate fra i disordini con maggior prevalenza in questo campo (Feigin *et al.*, 2017). Nonostante questo fardello sia diventato via via più pressante col tempo, ad oggi l'industria farmaceutica globale non è riuscita a proporre efficaci misure risolutive per l'ampio e diversificato spettro di patologie in questo settore. Come dimostra una recente analisi statistica, infatti, nel decennio 2006 – 2015 solo l'8.4% dei potenziali farmaci proposti nell'ambito neurologico ha incontrato un favorevole parere dell'FDA (Hay *et al.*, 2014). Se si considerano poi i soli candidati rientranti nella nomenclatura "Grandi Molecole" in tutti i settori, si denota che il 13.2% di essi ha una possibilità di essere accettato come reale farmaco. Al di là di misure di controllo talvolta eccessivamente restrittive, un tasso di successo così basso scaturisce sovente da un'inefficiente valutazione delle reali potenzialità dei composti proposti per lo sviluppo di farmaci in fase preclinica. Date, infatti, le scarse possibilità di approvazione finale da parte degli organi competenti, viene logico ritenere che solo quelle molecole che fin dalle prime fasi di sviluppo dimostrano un'inconfutabile efficacia e un chiaro e sicuro meccanismo di azione meritino di essere portate avanti nel processo. Un'eccezione in questo quadro sfavorevole è costituita da quelle proteine umane che generalmente vengono prodotte ed utilizzate in terapie sostitutive per il trattamento di patologie monogeniche. Dato il basso grado di novità di queste molecole, in genere la probabilità di essere rapidamente accettate alla fine del processo di valutazione del farmaco è più alta (Gorzelany and De Souza, 2013). In questi casi sono le procedure di produzione e formulazione finale del prodotto stesso le fasi più critiche dell'intero iter di sviluppo, data la natura delicata delle molecole in oggetto (Saccardo, Corchero and Ferrer-Miralles, 2016).

In questo lavoro sono descritte le strategie e le procedure sperimentali impiegate nel corso della produzione ricombinante di due proteine con potenziali applicazioni nel trattamento di patologie del sistema nervoso centrale (SNC). Entrambe hanno imposto diverse sfide inerenti ad alcuni degli aspetti sopra accennati e le varie soluzioni proposte per superarle sono riportate.

Capitolo 1: Produzione e purificazione di CNF1 per la sua caratterizzazione funzionale e terapeutica

La tossina CNF1 (Cytotoxic Necrotizing Factor 1) è una proteina di 1014 amminoacidi secreta da alcuni ceppi patogeni di *Escherichia coli* nel corso di infezioni urinarie, gastrointestinali e meningiti. Il meccanismo di azione di tale tossina prevede l'interazione con due recettori di membrana largamente diffusi in vari tessuti dell'organismo umano. Dopodiché, essa è internalizzata nella cellula mediante endocitosi ed il suo dominio catalitico è rilasciato nel citosol dopo uno specifico taglio proteolitico. In questo compartimento cellulare tale dominio catalizza una reazione di deammidazione a carico di un residuo di glutammina delle Rho GTPasi RhoA, Rac1

e Cdc42. Complessivamente, tale modifica irreversibile induce l'attivazione costitutiva e la successiva degradazione proteasomale di questi regolatori cellulari, così da alterarne i fisiologici livelli cellulari. Questo processo è sfruttato dai batteri patogeni per interferire con il sistema immunitario dell'ospite e per facilitare la loro penetrazione attraverso i tessuti cellulari. L'attività delle Rho GTPasi è, infatti, intrinsecamente legata alla produzione di citochine proinfiammatorie, alla regolazione dell'organizzazione del citoscheletro di actina, alla forma, alla motilità ed alla vitalità cellulare. Sulla base dell'analisi dei peculiari fenotipi indotti dalla trasduzione di CNF1 all'interno del SNC, un gruppo italiano ha proposto l'impiego di tale tossina per il trattamento sintomatologico di patologie e disordini neuronali, quali la malattia di Alzheimer, il morbo di Parkinson, la sindrome di Rett e l'epilessia. Nonostante gli interessanti studi preliminari condotti su modelli murini delle summenzionate patologie, un'estensiva e robusta analisi preclinica che attesti la sicurezza e l'efficacia di un potenziale farmaco fondato su questa molecola non è stato ancora condotto. Per di più, sia in prospettiva di validazione clinica che di eventuale produzione industriale è essenziale verificare la possibilità di ottenere il bioprodotto di interesse a livelli accettabili in termini di rese, purezza e stabilità. I protocolli di espressione e purificazione di CNF1 riportati in letteratura sono abbastanza deludenti in tal senso. In taluni casi è ravvisabile una notevole contaminazione del campione finale da parte di frammenti di degradazione della proteina in esame o di altre proteine provenienti dall'ospite di espressione. Questo problema è stato arginato facendo ricorso a procedure di purificazione particolarmente complesse e lunghe che constano anche di oltre dieci passaggi consecutivi con un notevole impatto sulle rese finali. Anche quando sono stati adoperati metodi di purificazione più semplici, come una singola cromatografia di affinità, non è stato comunque possibile raccogliere una quantità di proteina ricombinante superiore all'ordine del centinaio di µg per litro di coltura. Infine, nessun dato circa la stabilità nel tempo della proteina è mai stato riportato.

Lo scopo principale di questo lavoro è consistito, dunque, nella definizione di una procedura semplice e riproducibile per l'espressione ricombinante e la purificazione di CNF1 raggiungendo standard quantitativi e qualitativi prossimi a quelli di un processo industriale. La proteina così ottenuta sarebbe stata poi impiegata in una successiva caratterizzazione *in vitro* ed *in vivo* in un'ottica di una sua valutazione preclinica. I dati così ottenuti sarebbero stati essenziali per valutare se procedere o meno con lo sviluppo di un potenziale farmaco per il trattamento dei disordini del SNC.

Per il raggiungimento degli obiettivi prefissati, la proteina in esame è stata prodotta per via ricombinante nel citosol di un ceppo commerciale di *E. coli* BL21(DE3) inducibile da IPTG, fondendola ad un tag di 8 istidine, così da ottenere il costrutto denominato CNF1-H8. La definizione delle migliori condizioni di crescita e di espressione è stata essenziale per snellire la successiva procedura di purificazione e per preservare la proteina in forma prevalentemente integra. Nella fattispecie, un'induzione condotta ad una temperatura bassa per questo ospite mesofilo (15 °C) ha costituito l'espediente più utile per arginare la "violenza" tipica del sistema di induzione usato (basato sulla T7 RNA polimerasi dei sistemi pET) e per minimizzare i processi degradativi maggiormente ravvisabili a temperature più alte (da 20 °C in su). La buona qualità della produzione e i discreti livelli di accumulo registrati in fase di espressione (ravvisabili anche solo per SDS-PAGE), hanno consentito di ottenere circa 7 - 8 mg di proteina pura per litro di coltura (> 95 % del contenuto proteico totale) alla fine dei passaggi cromatografici impiegati. In particolar modo,

l'accoppiamento di una cromatografia di affinità (IMAC) con una ad esclusione molecolare è stato sufficiente per raggiungere gli standard produttivi prefissati. L'utilizzo del secondo passaggio cromatografico è stato essenziale non solo per incrementare significativamente la purezza del campione finale, ma anche per diminuirne l'eterogeneità in termini di aggregazione. Come dimostrato dalle analisi condotte ricorrendo al Dynamic Light Scattering (DLS), infatti, solo con l'eliminazione finale degli aggregati proteici è stato possibile ottenere un prodotto relativamente omogeneo (Pdi 0.275) preservabile a 4 °C per tempi lunghi. Le analisi per DLS insieme ai saggi di attività condotti utilizzando la proteina RhoA come substrato sono state anche utili per giungere alla definizione di una formulazione stabile nel tempo. In particolar modo, per poter essere conservato a concentrazioni piuttosto alte (10 mg/mL), CNF1-H8 ha richiesto l'utilizzo di un tampone con una forza ionica relativamente alta (almeno 150 mM NaCl) e di 15% v/v glicerolo come co-solvente. Fra i due fattori il ricorso ad una elevata concentrazione salina durante la conservazione è risultato essere il più determinante sia per la preservazione dell'attività enzimatica che per il mantenimento di una condizione di quasi monodispersione nel tempo. Per di più, i saggi di intossicazione *in vitro* eseguiti su colture di cellule HEp-2 hanno confermato che alla fine della procedura di purificazione la proteina era correttamente strutturata per poter essere internalizzata e processata nelle cellule eucariotiche ed esplicare la sua attività catalitica. Seppure alcuni componenti della soluzione tampone di preservazione avrebbero potuto costituire di per se stessi una causa di tossicità, si deve considerare che le preparazioni erano così concentrate che la diluzione nella soluzione del saggio finale ha reso ininfluente il loro contributo. Infine, per la prima volta è stata condotta una generica caratterizzazione strutturale della tossina CNF1-H8 nella sua interezza, ricorrendo ad un'indagine di dicroismo circolare (CD). Lo spettro ottenuto nel lontano UV ha confermato i dati pregressi di predizione di strutture secondarie, indicando che circa il 50% della proteina è molto flessibile e questo probabilmente costituisce il maggior scoglio per la definizione della struttura tridimensionale dell'intera tossina. Differentemente dalle analisi per DLS, gli studi di stabilità termica condotti con il CD hanno inoltre suggerito che la proteina potrebbe essere più stabile ad un pH lievemente acido (pH 6.6), dando ulteriori spunti per successive condizioni da saggiare per la preservazione della molecola.
Nonostante le buone *performance* raggiunte nel processo produttivo, lo sviluppo di CNF1-H8 od un suo derivato come farmaco non è stato portato avanti. In assenza di un'esauriente e puntuale validazione in termini di *Target Engagement*, *Proof of Mechanism*, *Proof of Principle* e *Proof of Concept*, proseguire il progetto avrebbe costituito un notevole rischio in termini economici e di opportunità. Nonostante tutto, le comprovate riproducibilità e semplicità dei protocolli di produzione impiegati potrebbero costituire una buona base per l'ulteriore caratterizzazione della proteina in chiave tossicologica oppure per il suo impiego come immuno-adiuvante.

Capitolo 2: Produzione ricombinante di una proteina coinvolta nello sviluppo cerebrale in *Pseudoalteromonas haloplaktis* TAC125

La seconda parte di questo lavoro di tesi ha riguardato la produzione di una proteina umana (in questo testo denominata Variant B) per la sua caratterizzazione ed il suo impiego in una terapia sostitutiva. Le informazioni relative al funzionamento di questa molecola sono ancora piuttosto lacunose anche se un'intensa ricerca è in corso in vari centri per studiarne le implicazioni nello sviluppo cerebrale. Infatti, seppure diverse isoforme di Variant B sono espresse in molteplici tessuti cellulari, è evidente

che la sua subnormale espressione nel SNC comporta i più gravi deficit fenotipici durante lo sviluppo infantile. Studi preclinici in modelli murini hanno dimostrato che una terapia proteica sostitutiva può effettivamente ripristinare un fenotipo sano in soggetti affetti da mutazioni aberranti a carico del gene *variant B.* Nonostante ciò, una cura non è ancora praticabile date le grandi difficoltà nel produrre la proteina per via ricombinante con le piattaforme di espressione più canoniche.
Il gruppo di ricerca in cui ho condotto questo lavoro di tesi si è quindi proposto di impiegare un batterio non convenzionale per tentare l'espressione di Variant B. Si tratta di *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125), un γ-proteobatterio antartico marino ampiamente caratterizzato da un punto di vista genetico e metabolico e la cui utilità biotecnologica è stata dimostrata in vari ambiti. In particolar modo, mediante la fusione dell'origine di replicazione del plasmide endogeno pMtBL di *Ph*TAC125 a frammenti di plasmidi di *E. coli* è stata sviluppata una serie di vettori di clonaggio e di espressione da poter utilizzare nel batterio polare. In questo modo, più proteine eucariotiche sono state sintetizzate per via ricombinante in *Ph*TAC125 a partire da questi vettori navetta dimostrando il loro efficace impiego per la produzione di proteine "difficili".

Summary

Neurological disorders constitute the major cause of disability adjusted-life years (DALYs). Alzheimer's disease and other dementias are included in the group of the most prevalent disorders in this field (Feigin *et al.*, 2017). Nevertheless, the urgency of the treatment of these pathologies is not met by efficacious drug development, as indicated by a recent statistical analysis measuring the likelihood of approval of new drugs by disease area (Hay *et al.*, 2014). In neurology only 8.4% of the candidate drugs have been approved in the 2006 – 2015 decade and the drugs categorized as "Large Molecules" were characterized by 13.2% rate of success (Hay *et al.*, 2014). This statistical analysis would suggest that only those molecules proving robust Proof of Mechanism (PoM) and Proof of Principle (PoP) during their early development deserve the risk for further development. On the other hand, proteins used in protein replacement therapies (PRTs) constitute an exception in this scenario as the *iter* for their approval is generally more straightforward (Gorzelany and De Souza, 2013). In this case the main hurdles involving the drug development directly coincide with the production, the purification and the stable formulation of the final product rather than the assessment of its efficacy or toxicity (Saccardo, Corchero and Ferrer-Miralles, 2016).

In the present document we describe the approaches followed in the implementation of biotechnological processes for the production of two different proteins with potential applications in the treatment of central nervous system (CNS) disorders. In the first chapter we present the establishment of a simple and efficient pipeline for the *E. coli* recombinant expression and purification of the bacterial toxin named CNF1. This 114 kDa protein is involved in a series of infectious diseases (Ho *et al.*, 2018), but it has also been demonstrated to be promising in the treatment of severe neurological pathologies like Alzheimer's disease, Parkinson disease, Rett syndrome and epilepsy (Maroccia *et al.*, 2018). Nevertheless, during the development of the project we decided not to pursue any further attempt in the clinical development of CNF1 as a drug because of the lack of robust, clear and completely demonstrated PoM and PoP. However, the proposed procedure for the purification and final formulation of the product outperforms the others reported in the literature in terms of yield, purity and stability and it can be easily employed in the future for further structural and functional analyses in toxicological and immunological perspectives. The reproducibility of the entire pipeline has been demonstrated repeating the production and purification protocols dozens of times at intervals of several months. Moreover, the stability of the final product was routinely ascertained using SDS-PAGE, size-exclusion chromatography, DLS and activity assays.

In the second chapter of this thesis we describe the employment of *Pseudoalteromonas haloplanktis* TAC125 in the production of a human protein to be used in a PRT. Although the quality of the product achievable in this host seemed better than the one previously obtained with other expression systems, the overall yields remained low. Slight improvements in this sense were achieved by the genetic engineering of the coding sequence of the target protein and by the implementation of alternative expression plasmids. Nevertheless, further studies demonstrated that the whole expression platform – the host and the plasmids – are affected by imperfections and bottlenecks whose correction is pivotal for a satisfying recombinant production. This kind of issues is typical of unconventional and less explored recombinant bacteria, but there are several examples in the literature about how they can be systematically overcome. Hence, a series of measures to be taken for the improvement of this microbial factory are proposed.

Chapter 1: CNF1 production and purification for its functional and therapeutic characterization

1.1 Introduction

1.1.1 CNF1 toxin as a prototypical virulence factor targeting Rho GTPases

"Cytotoxic Necrotizing Factor 1" takes its name from the observation that its inoculation in eukaryotic cell cultures induces cellular multinucleation and its subcutaneous injection provokes the appearance of skin necrotic lesions (Caprioli *et al.*, 1983, 1984). Over the years, CNF1 has become the most studied representative of a growing family of tightly similar toxins that includes at least nine close homologs secreted by different bacteria for the intoxication of different hosts (Ho *et al.*, 2018). However, CNF1 is exclusively produced by some pathogenic *Escherichia coli* strains mainly involved in urinary tract infections, intestinal infections and meningitis in humans (Falbo *et al.*, 1993; Khan *et al.*, 2002). In such disparate tissues this bacterial protein interferes with the functioning of Rho GTPases, master regulators of cellular homeostasis, with the overall effect of increasing the colonizing properties of the producing strains (Fiorentini *et al.*, 1997; Flatau *et al.*, 1997; Schmidt *et al.*, 1997; Lerm *et al.*, 1999; Doye *et al.*, 2002). Rho GTPases are indeed targets of various bacterial effectors due to their involvement in a series of processes which can be exploited by pathogens to potentiate their invasion (Boquet and Lemichez, 2003; Galan, 2009; Lemichez and Aktories, 2013). Depending on the mechanism of action, the breach of Rho GTPases regulated processes by toxins can lead to: 1) disruption of host barriers; 2) invasion of non-phagocytic cells; 3) interference with the immune system. Actually, thanks to its sneaky mode of action, CNF1 acts on all the three abovementioned phenomena.

CNF1 is a 1014 aa AB toxin which, after secretion, enters mammal cells by the binding with the laminin receptor precursor 67LR together with the Lutheran adhesion glycoprotein/basal cell adhesion molecule (Lu/BCAM, Lemichez *et al.*, 1997; Fabbri, Gauthier and Boquet, 1999; Chung *et al.*, 2003; Kim, Chung and Kim, 2005b; McNichol *et al.*, 2007; Piteau *et al.*, 2014). Then it triggers the constitutive activation and induces the proteasomal degradation of Rho GTPases by deamidation of a glutamine residue (Gln 63 in RhoA, Gln 61 in Cdc42 and Rac1, Fiorentini *et al.*, 1997; Flatau *et al.*, 1997; Schmidt *et al.*, 1997; Lerm *et al.*, 1999; Doye *et al.*, 2002). The mechanism of internalization of this toxin is quite complex and requires its binding with both the receptors, its transport via endocytosis and the release of the cleaved C-terminal domain in the cytosol after an acidic-dependent insertion of two hydrophobic regions in the endosomal membrane (Contamin *et al.*, 2000; Pei, Doye and Boquet, 2001; Doye *et al.*, 2002, Figure 1.1).

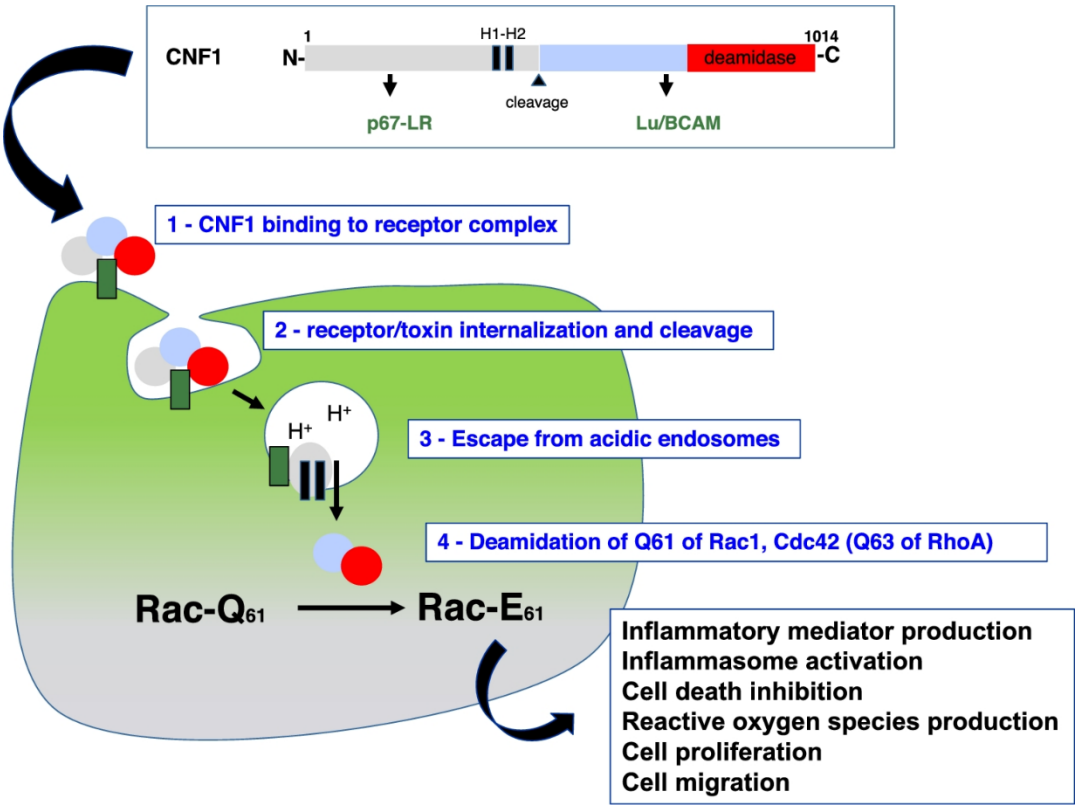

Figure 1.1. Schematic representation of CNF1 functional domains and its mechanism of action. CNF1 is a 1014 aa protein belonging to the AB toxin category. At least two recognition sequences have been identified to interact with laminin receptors: residues 1-342 binding with p67-LR and residues 709-730 with Lu/BCAM. After receptor-mediated endocytosis (1) and endosome acidification, the insertion of two hydrophobic helices (H1 and H2) in the endosomal membrane allows the cleavage (2) and the release in the cell cytosol of a 55 kDa fragment encompassing the catalytic domain (deamidase). Here the C-terminal domain deamidates Rho GTPases specific residues (4), triggering a series of cellular effects summed up in the figure. Taken from Ho *et al.*, 2018.

Although the process of activation and the following proteolysis of the effector proteins might seem a contradiction in the perspective of a bacterial invasion, this temporal switch makes sense in the context of a mechanism of defense exploited both by the host and the pathogen for their preservation. One of the main effects of Rho GTPases activation involves a wide actin cytoskeletal reorganization that is translated in the formation of different ultrastructures, depending on the predominantly activated protein: stress fibers (RhoA), lamellipodia (Rac1), filopodia (Cdc42) (Boquet and Lemichez, 2003). In any case such rearrangements deeply affect cellular polarization, motility and the integrity of cellular junctions, leading to an increased permeabilization of endothelial barriers (Lemichez and Aktories, 2013). At the same time (Fiorentini *et al.*, 2001), the induction of the formation of extracellular protrusions facilitates unspecific phagocytosis and macropynocitosis of extracellular material by unspecialized cells. Moreover, CNF1 induces cellular proliferation thanks to an altered expression of Bcl-2 family proteins (Fiorentini *et al.*, 1998) via the AKT/NF-kB regulatory pathway (Miraglia *et al.*, 2007). All in all, these cellular responses are used to disrupt endothelial barriers and facilitate intracellular migration of the pathogens. Nevertheless, an uncontrolled Rho GTPases hyperactivation would be detrimental both for the host and the pathogen because it would provoke excessive tissues alterations. As G proteins are canonical target of many bacterial

effectors and their dysregulation leads to important alterations in cellular homeostasis, mammals have evolved a defense mechanism, through which a strong immune response is triggered when Rho proteins are abnormally activated (Boquet and Lemichez, 2003). Nevertheless, an uncontrolled defense can become harmful even for the host, as it might lead to damages to its own “hyperactivated” tissues. That is why, eukaryotic cells recognize postranslationally modified and constitutively activated Rho GTPases as aberrant proteins and shunt them towards ubiquitination and degradation, so that the violent immune alarm is shut down (Lemonnier, Landraud and Lemichez, 2007). As a result of this sophisticated mechanism of host-pathogen interaction, CNF1 intoxication often leads to a mild activation of Rho proteins (among which RhoA seems the predominant) that triggers a controlled immune response which is neither harmful for the host nor extremely efficient to defeat the pathogen (Munro *et al.*, 2004). The systematic exploitation of these complex endogenous regulatory mechanisms by pathogens to bypass the immune response is further confirmed by the existence of redundant and counteracting bacterial effectors, like hemolysin whose function, among the others, seems to consist in the reduction of the production of cytokines induced by CNF1 action (Diabate *et al.*, 2015). In other words, even if during the evolution these pathogens became detectable by the immune system because of CNF1 action, they managed to preserve this useful toxin by evading the host defense both releasing damage control co-effectors and exploiting the repair mechanisms of the host itself.
This general overview of the mode of action of this family of toxins highlights how the subtle alteration of important homeostatic processes resulting from host-pathogen crosstalk are crucial both for bacterial survival and for the preservation of the host. Paradoxically, if properly exploited, these same processes might be used to produce positive effects in the host, as explained in the following paragraph.

1.1.2 CNF1 toxin as a drug candidate for the treatment of CNS disorders

As discussed in the previous section, the main direct effects of the action of CNF1 on Rho GTPases consist of cytoskeletal reorganization (Boquet and Lemichez, 2003) and the activation of phosphorylation regulatory pathways influencing many processes, including mitochondrial homeostasis (Miraglia *et al.*, 2007). Some both chronic and acute diseases affecting the brain are characterized by common traits, including neuronal atrophy, poor plasticity, and bioenergetics issues (Beal, 2004; Wilkins *et al.*, 2014; Carvalho *et al.*, 2015). Hence, an Italian research group working at Istituto Superiore di Sanità proposed the use of CNF1 to relieve the symptoms of such diseases, proving its efficacy first in the treatment of Rett syndrome (De Filippis *et al.*, 2012; De Filippis, Valenti, Chiodi, *et al.*, 2015; De Filippis, Valenti, De Bari, *et al.*, 2015) and, then, in Alzheimer’s disease (Loizzo *et al.*, 2013) and Parkinson’s disease (Musilli *et al.*, 2016) in mice models. The molecular reasons that are at the basis of these explorative and preliminary results are effectively summarized in a recent review (Maroccia *et al.*, 2018) and in Figure 1.2. Basically, the cytoskeletal reorganization induced by activated Rho GTPases is capable of reverting astrocytes atrophy and to restore their supportive role on neurons (Malchiodi-Albedi *et al.*, 2012). On the other side, the activation of the cAMP/PKA phosphorylation cascade directly influences mitochondria functioning, both by inhibiting their fission and by directly regulating proteins involved in the respiratory chain (Travaglione *et al.*, 2014). This same last process proved to be promising in the treatment of epilepsy (Fabbri *et al.*, 2018) that is characterized by brain mitochondrial misfunction. Finally, CNF1

capability to alter the normal cell cycle progression was demonstrated to be successful in the treatment of glioma (Vannini *et al.*, 2014).

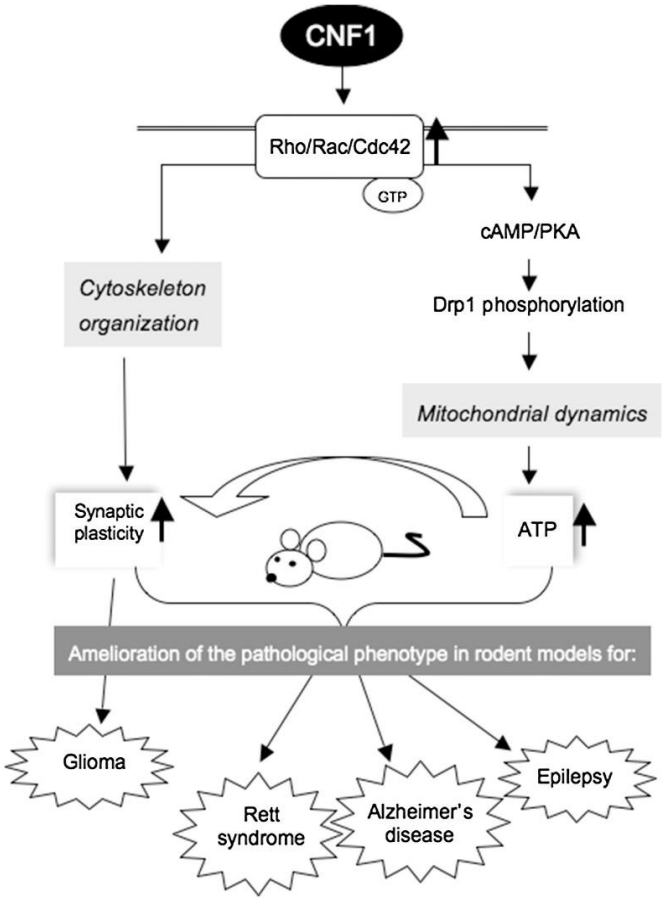

Figure 1.2. Schematic outline of CNF1 therapeutic features in animal models recapitulating CNS diseases. Rho GTPases functioning is perturbed by CNF1 catalyzed deamidation and proteasome degradation. This is translated in the induction of a profound actin reorganization of the intoxicated cells (left side) and in the triggering of a phosphorylation cascade started by cAMP accumulation (right side). Cytoskeletal alteration is useful to increase synaptic plasticity and revert astrocytes atrophy in CNS diseases on one side, and to block the proliferation of cancer cells on the other. The activation of the PKA phosphorylation cascade ameliorates the cellular bioenergetics, allowing both an increase of ATP production and a reduction of ROS. This result is mainly due to the inactivation of the mitochondrial profission Drpl protein. Taken from Maroccia *et al.*, 2018.

All the above-mentioned proof-of-concept studies that have been carried out in the last decade show how CNF1 action on Rho GTPases is useful to interfere with some CNS diseases and brain functioning in general. Nevertheless, little has been done to investigate its real applicability as a therapeutic drug so far. This section of the present document aims to give a small contribution in this sense, both by providing a protocol for the production and purification of the toxin and some considerations about its use.

1.2 Aim of the study

This project started as a collaboration with Dr. C. Fiorentini and A. Fabbri working at Istituto Superiore di Sanità and it aimed to evaluate the feasibility of the use of CNF1 toxin as a protein drug in the treatment of CNS disorders. It was supposed to consist of four main phases:

1) The establishment of a simple procedure for the recombinant production and purification of the *wt* protein.
2) The evaluation of the fundamental pharmacodynamics (PD) and pharmacokinetics (PK) parameters using different routes of administration, like bioavailability, distribution, efficacy, toxicity, etc.
3) The rational design of CNF1 variants suitable for systemic administration on the base of the PD and PK preliminary data and of the already existing structural/functional information.
4) Characterization of the engineered CNF1 variants.

Because of the lack of collaboration of our partners, the project never moved further than the first step.

1.3 Results

The results of this section are reported in the following published article:

Colarusso, A., Caterino, M., Fabbri, A., Fiorentini, C., Vergara, A., Sica, F., Parrilli, E. and Tutino, M. L. (2018), High yield purification and first structural characterization of the full-length bacterial toxin CNF1. Biotechnol Progress, 34: 150-159. doi:10.1002/btpr.2574.

High Yield Purification and First Structural Characterization of the Full-Length Bacterial Toxin CNF1

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The Cytotoxic Necrotizing Factor 1 (CNF1) is a bacterial toxin secreted by certain Escherichia coli *strains causing severe pathologies, making it a protein of pivotal interest in toxicology. In parallel, the CNF1 capability to influence important neuronal processes, like neuronal arborization, astrocytic support, and efficient ATP production, has been efficiently used in the treatment of neurological diseases, making it a promising candidate for therapy. Nonetheless, there are still some unsolved issues about the CNF1 mechanism of action and structuration probably caused by the difficulty to achieve sufficient amounts of the full-length protein for further studies. Here, we propose an efficient strategy for the production and purification of this toxin as a his-tagged recombinant protein from* E. coli *extracts (CNF1-H8). CNF1-H8 was expressed at the low temperature of 15°C to diminish its characteristic degradation. Then, its purification was achieved using an immobilized metal affinity chromatography (IMAC) and a size exclusion chromatography so as to collect up to 8 mg of protein per liter of culture in a highly pure form. Routine dynamic light scattering (DLS) experiments showed that the recombinant protein preparations were homogeneous and preserved this state for a long time. Furthermore, CNF1-H8 functionality was confirmed by testing its activity on purified RhoA and on HEp-2 cultured cells. Finally, a first structural characterization of the full-length toxin in terms of secondary structure and thermal stability was performed by circular dichroism (CD). These studies demonstrate that our system can*

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be used to produce high quantities of pure recombinant protein for a detailed structural analysis. © 2017 American Institute of Chemical Engineers *Biotechnol. Prog.*, 000:000–000, 2017

Keywords: CNF1, recombinant protein production, protein purification, circular dichroism, secondary structure analysis

Introduction

The Cytotoxic Necrotizing Factor 1 (CNF1) is a 1014 aa toxin produced and secreted by some *Escherichia coli* pathogenic strains principally involved in urinary tract infections and neonatal meningitis.[1,2] Although the understanding of CNF1 direct role in bacterial infection is complicated by the overlap with the action of other bacterial effectors,[3–5] it has been extensively demonstrated that this toxin triggers the constitutive activation and induces the subsequent proteasomal degradation of Rho GTPases by deamidation of a glutamine residue (Gln63 in RhoA, Gln61 in Cdc42 and Rac).[6–10] The main effects of this process include the activation of a controlled immune response in the host[11] and a wide reorganization of the actin cytoskeleton of the infected cell which is exploited for the bacterial uptake.[12,13] CNF1 capability to directly affect Rho GTPases action and indirectly actin assembly and cellular bioenergetics has recently been exploited in the treatment of some severe neurological diseases, including Alzheimer's disease, Parkinson's disease and Rett syndrome.[14–22]

Several studies have pointed out the general structuration of CNF1 as an AB toxin, presenting two different receptor-recognition sites (an N-terminal 37LRP/67LR binding domain and a C-terminal Lu/BCAM binding domain) and a C-terminal catalytic domain catalyzing Rho deamidation.[23–28] The mechanism of internalization of this toxin is quite complex and requires its binding with both receptors, its transport via endocytosis and the release of the cleaved C-terminal domain in the cytosol after an acidic-dependent insertion of two hydrophobic regions in the endosomal membrane.[28–30] A great piece of information about the CNF1 mechanism of function and modular structuration derived from a comparison with other well-known toxins such as the diphtheria toxin and the dermonecrotic toxin.[31] This homology-based hypothesis has been further corroborated by a series of *in silico* prediction studies,[1,29] mutagenesis experiments[29,30] and interactome analysis[25,28] so as to have the general idea of CNF1 structure as described above. Nevertheless, only the C-terminal catalytic domain of the protein (residues 720–1014) has been deeply studied from a structural point of view thanks to the work of Buetow et al. who have determined the X-ray crystal structure of this domain[32] and its critical structural constrains for the catalyzed reaction.[33]

We think that such a detailed level of information should be extended to the remaining predominant part of the protein to achieve a clear understanding of its mechanism of action and its potential therapeutic use. In fact, there are still too many unsolved questions about CNF1 function. For example, why the binding with two different receptors is needed for the toxin internalization into eukaryotic cells? What are the roles of the two receptors? What does CNF1 conformational change experienced during endosomal acidification consist of? Which protease cleaves and provokes the release of the CNF1 catalytic domain in the cell cytosol? More importantly, the use of CNF1 as a therapeutic for the treatment of neurological disorders demands an unbiased evaluation of its intrinsic risks that can be fully contemplated only in the presence of data about the protein structure and function.

We think that the lack of structural studies about the full-length CNF1 issues from the difficulty to recombinantly produce and purify it with high-yield in a simple way. Various research groups reported to still use the protocol established by Falzano et al.[12] which is long, complex, and leads to the harvest of insufficient amounts of protein. Particularly, this procedure consists of over ten steps including three ammonium sulphate precipitations alternated with dialysis and centrifugations, two ion exchange chromatographies, two gel filtrations and one hydrophobic exchange chromatography.[12] Other groups testified a remarkable instability of the protein that was progressively proteolysed during its synthesis and difficult to separate from its degradation products;[27,34] Schmidt's research group routinely produces CNF1 as a GST-fused chimera with a reported yield of purified toxin of 1 mg per 4 liters of culture, but this yield remains unsatisfying for a complete chemical-physical characterization.[35]

In this study, we propose a procedure aiming to increase both CNF1 production and purification yields, and to minimize its instability and degradation, for collecting sufficiently pure, concentrated and homogeneous CNF1.

Materials and Methods

Construction of pET40b-CNF1-H8 expression plasmid

The *cnf1* gene was inserted into the pET40b expression vector in three fragments. The central fragment of the gene (2150 bp) was obtained with *BglII*/*NcoI* double digestion of the pISS392 plasmid.[1] The 5′ part (190 bp) and the 3′ part (740 bp) of the sequence were synthesised by Thermo Fisher Scientific and digested with *NdeI*/*BglII* and *NcoI*/*XhoI* restriction enzymes, respectively. The three fragments were then consecutively cloned into the pet40b plasmid between *NdeI* and *XhoI* restriction sites. The so obtained pET40b-*cnf1*-H8 vector allowed the production of CNF1-H8, a protein encompassing the wild type 1014 residues fused to an 8xHis C-terminal tag encoded by the plasmid sequence.

Expression and purification of CNF1-H8 recombinant protein

pET40b-*cnf1*-H8 vector was introduced into *E. coli* BL21(DE3) cells and different conditions were tested to assess CNF1-H8 expression. M9, LB, and TB[36] and their variants were used as different growth media. Particularly, different carbon sources (glucose, glycerol, acetate -each added at 0.4–1.0% w/v range-, and their combinations) were tested to define their effect on protein production quality.[37–39] IPTG induction was performed in different growth phases in a 0.1–1 mM range. Finally, 15°C, 20°C, and 37°C were the explored temperatures during the growth and production phases. Once these different conditions had been tested, the production of the recombinant protein was routinely performed growing the

Table 1. List of Buffers and Additives Used During CNF1-H8 Storage and DLS Analysis at 4°C

Tested buffers	Tested additives
50 mM TrisHCl pH 8.0	5–30 % v/v Glycerol
PBS pH 7.4	5–30 % v/v Glucose
100 mM carbonate buffer pH 9.3	20–500 mM NaCI
50 mM Bis-tris pH 6.5	
8 mM Sodium Phosphate pH 6.6–7.8	

bacteria in LB medium in the presence of 50 μg/mL of kanamycin at 20°C. At $OD_{600} = 0.8$, IPTG was supplemented to the final concentration of 0.5 mM and the temperature was shifted to 15°C overnight.

After recombinant protein production in the chosen conditions, the bacterial cells were harvested by centrifugation (4500*g*, 4°C, 30 min) and washed with phosphate-buffered saline (PBS). Then the pellets were resuspended in 5 mL/g wet cell weight of Lysis buffer (50 mM TrisHCl pH 8.0, 150 mM NaCl, 20 mM Imidazole, 15% v/v glycerol) supplemented with the complete EDTA-free protease inhibitor cocktail (Roche). Afterwards, the suspensions were subjected to disruption by sonication (30 s cycles, 25% Amplitude with 30 s pauses between each cycle for a total process of 20 min at 4°C) and the soluble and insoluble fractions were separated by centrifugation (13,000*g*, 4°C, 45 min) and filtration.

The clarified lysates were applied to IMAC using a 1 mL HisTrap HP column (GE Healthcare) and an Akta purifier system (GE Healthcare). The bound fractions were washed and eluted using IMAC Wash buffer (50 mM TrisHCl pH 8.0, 150 mM NaCl, 50 mM Imidazole, 15% v/v glycerol) and IMAC Elution buffer (50 mM TrisHCl pH 8.0, 150 mM NaCl, 500 mM Imidazole, 15% v/v glycerol), respectively. The purity of the eluted fractions was further increased by a size exclusion chromatography (Superdex 200 pg, GE Healthcare) performed using 50 mM TrisHCl pH 8.0, 200 mM NaCl, 15% v/v glycerol as running buffer at 0.5 mL/min flow rate. At the end of the procedure, the recombinant proteins were dialyzed and concentrated (up to 10 mg/mL) in Storage buffer (50 mM TrisHCl pH 8.0, 200 mM NaCl, 30% v/v glycerol, 2 mM DTT) and preserved at −20°C.

Throughout the expression and purification procedures, CNF1-H8 preparations were separated by 10% SDS-PAGE and analyzed both by Coomassie staining and Western blot using a monoclonal anti-CNF1 antibody (NG8, Santa Cruz Biotechnology, 1:1,000 dilution) and a secondary anti-mouse antibody (1:10,000 dilution). Their concentration was assessed using the Bradford Protein Assay following the manufacturer's instruction (Bio-Rad).

Dynamic light scattering (DLS)

The analytes frozen in Storage buffer at 10 mg/mL were diluted to 1 mg/mL in the chosen buffer and further equilibrated performing a diafiltration with Vivaspin 500 centrifugal concentrators (10 kDa, Sartorius) with two washes. In case of apparent precipitation, the suspensions were centrifuged and the soluble fractions were analyzed. The dispersity (Polydispersion index, Pdi) and the particles size were determined at 25°C performing four runs (15 measurements each) with a Zetasizer Nano ZS (Malvern Instruments Limited, Malvern, Worcestershire, UK). Table 1 presents the list of different buffers and additives which were exploited to explore the protein stability in different storage conditions.

RhoA recombinant production, purification and SDS-urea PAGE mobility assay

RhoA was produced and purified following the protocol described by Self et al.[40] using the pGEX2T-N25RhoA vector (kindly provided by E Lemichez, INSERM, Nice, France). Briefly, *E. coli* TOP10 bearing the pGEX2T-N25RhoA vector were induced with 0.1 mM IPTG at 20°C for 16 h. After centrifugation, cells were lysed in 50 mM TrisHCl pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT. GST-RhoA was purified using a GSTrap HP column (GE Healthcare) using 10 mM reduced glutathione during the elution. After dialysis in Cleavage/Assay buffer (50 mM TrisHCl pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM DTT), Thrombin (Novagen) was added to the sample (2U per mg of protein) for 20 h at 20°C. GST was removed using a second glutathione affinity chromatography and RhoA was further purified by size exclusion chromatography (Superdex 75 pg, GE Healthcare). Around 50 mg of pure protein was collected from a 1 L culture and it was finally stored in the Cleavage/Assay buffer at −20°C at a concentration of 1 mg/mL.

For *in vitro* mobility assays, recombinant RhoA protein (10 μM) was mixed with CNF1-H8 (0.2 μM) and incubated in 50 mM TrisHCl pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1mM DTT for increasing times at 25°C. The reaction was stopped by the addition of Laemmli buffer, the mixture was then subjected to SDS-PAGE on a 12.5% gel with 1M Urea and the proteins were detected by Coomassie staining. The deamidated form of RhoA (RhoA E63) generated by CNF1-H8 action showed a delayed electrophoretic mobility over its unmodified form (RhoA Q63), as reported in refs.[6,33]

Multinucleation assay

Human epithelial HEp-2 cells (ATCC® CCL23™) were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (Flow Laboratories, Rockville, MD, USA), 5 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were seeded at a density of 2×10^4 cells/cm^2 in p24 well plates. Twenty-four hours after seeding, cells were exposed to serial dilution of CNF1-H8, starting from 10^{-7} M to 10^{-16} M. For the activity assay, the percentage of multinucleated cells was counted at each dilution and the minimal dose causing multinucleation in 50% of cells was identified.

Circular dichroism (CD)

CD spectra of CNF1-H8 have been collected by using a Jasco J-710 spectropolarimeter equipped with a Peltier thermostated cell holder (Model PTC-348WI) (Jasco, Easton, MD). The mean residue molar ellipticity, $[\theta]$ in deg cm^2 dmol^{-1}, has been calculated from:

$$[\theta] = [\theta]_{obs} \times MRW/(10cl)$$

Where $[\theta]_{obs}$ is the measured ellipticity in degrees, MRW the mean residue molecular weight, c the protein concentration in g mL^{-1} and l is the optical path length in cm. Far-UV measurements (190–250 nm) have been performed by using a 0.1 cm

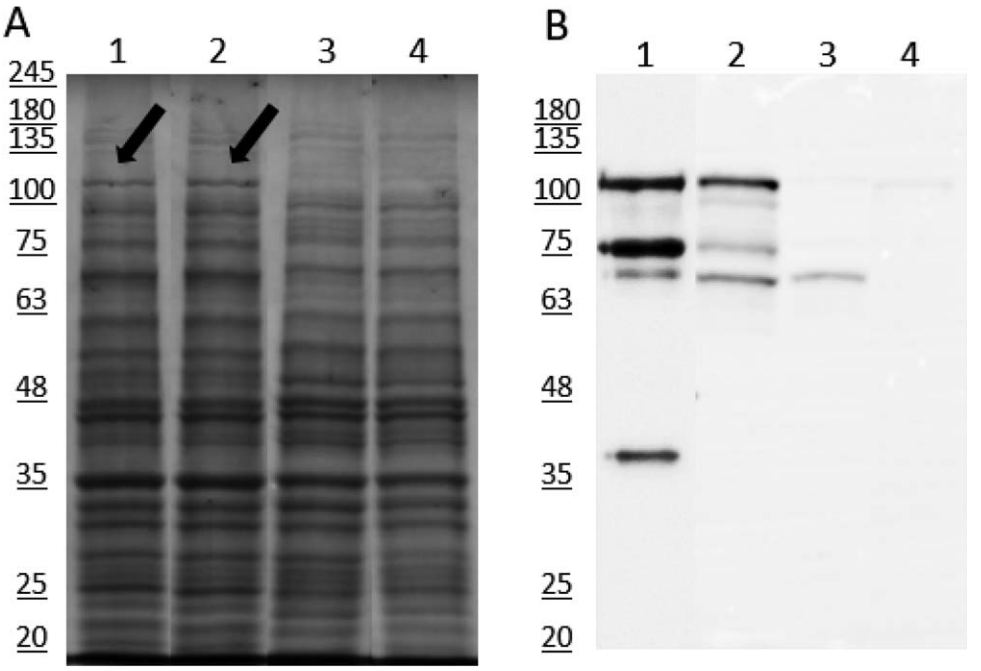

Figure 1. CNF1-H8 expression levels *in E. coli* BL21(DE3). Whole cell lysates were evaluated by SDS-PAGE (A) and Western blot analysis (B). 1, Induced cell lysate at 20°C after overnight expression; 2, induced cell lysate after temperature downshift to 15°C and overnight expression; 3, induced cell lysate at 37°C after 6 h expression; 4, uninduced cell lysate. Full-length CNF1-H8 is indicated with an arrow.

cell and protein concentration 1 nM. Thermal unfolding curves have been recorded in the temperature mode at 209 nm between 20 and 70°C. Temperature ramp 0.5°C min^{-1} and 60 s equilibration time prior each record have been used. Data points have been collected thrice hence averaged. Data has been fitted by mean of Boltzmann sigmoid equation:

$$y = \frac{A_1 - A_2}{1 + e^{(x - x_0)/dx}} + A_2$$

Spectra deconvolution and secondary structure evaluation have been performed using Dichroweb server,[41,42] by mean of either CONTIN[43] or SELCON3[44] method (best matches achieved with reference sets 4 and 3 respectively).[45,46]

Results and Discussion

Production and purification of recombinant CNF1-H8

CNF1-H8 protein was produced *in E. coli* using a T7-based expression vector (pET40b). This system allowed the production of a His-tagged recombinant protein to simplify the purification procedures. Nevertheless, as already experienced by other research groups,[34] CNF1-H8 overexpression in *E. coli* was characterized by the progressive release of some degradation products, probably due to proteolytic attack and its intrinsic instability. To face this issue, variables like growth medium composition, temperature, inducer concentration and duration of induction were taken into account (see *Materials and Methods* section). The variation of either induction profile or medium composition did not significantly affect CNF1-H8 production quality (data not shown). Conversely, the selected temperature during the growth and induction phases seemed to be crucial. Figure 1 shows the SDS-PAGE (A) and Western blot (B) analysis of bacterial extracts collected after *E. coli* growth in the presence (lanes 1–3) and absence (lane 4) of IPTG induction. In particular, lane 1 is representative of a bacterial culture grown at 20°C until mid-exponential phase ($OD_{600} = 0.8$) and induced overnight with 0.5 mM IPTG keeping the temperature constant. The condition represented by lane 2 just differed from the previous one in the use of a temperature downshift to 15°C after induction. In lane 3, instead, a lysate obtained after a 6 h expression at 37°C was loaded. The 116 kDa band characteristic of full-length CNF1-H8 was distinguishable in induced lysates in both panels mainly when the expression was performed at low temperatures (highlighted with an arrow), while at 37°C the soluble protein was mostly proteolyzed. The Western blot detection revealed the co-presence of some degradation products also when the expression was performed at suboptimal temperatures (Figure 1B). Nonetheless, at 15°C the intact protein resulted in the most prominent signal in comparison to its degradation products in the same lane (lane 2 in Figure 1), indicating that the temperature downshift was useful to stem CNF1-H8 proteolysis, which was significantly more evident in the other experimental conditions. The expression performed at 20°C gave the best result in terms of absolute recombinant protein production, as shown in lane 1 in Figure 1, but the degradation processes were also more significant. Considering that the NG8 Mab binds CNF1 C-terminal portion, the proteolyzed fragments highlighted in this Western blot are likely to preserve the 8xHis C-terminal tag and to be co-purified with the full-length protein during IMAC. In this way, it would have been quite complex to isolate the intact protein over its degradation products, representing more than the 50% of the produced CNF1-H8. That is why, we generally preferred to induce the production of the recombinant protein at 15°C rather than 20°C.

As reported in the *Introduction* section, the currently available protocols for CNF1 purification are either quite complex and time-consuming[12] or they lead to the collection of a final product whose quality and/or quantity is unsuitable

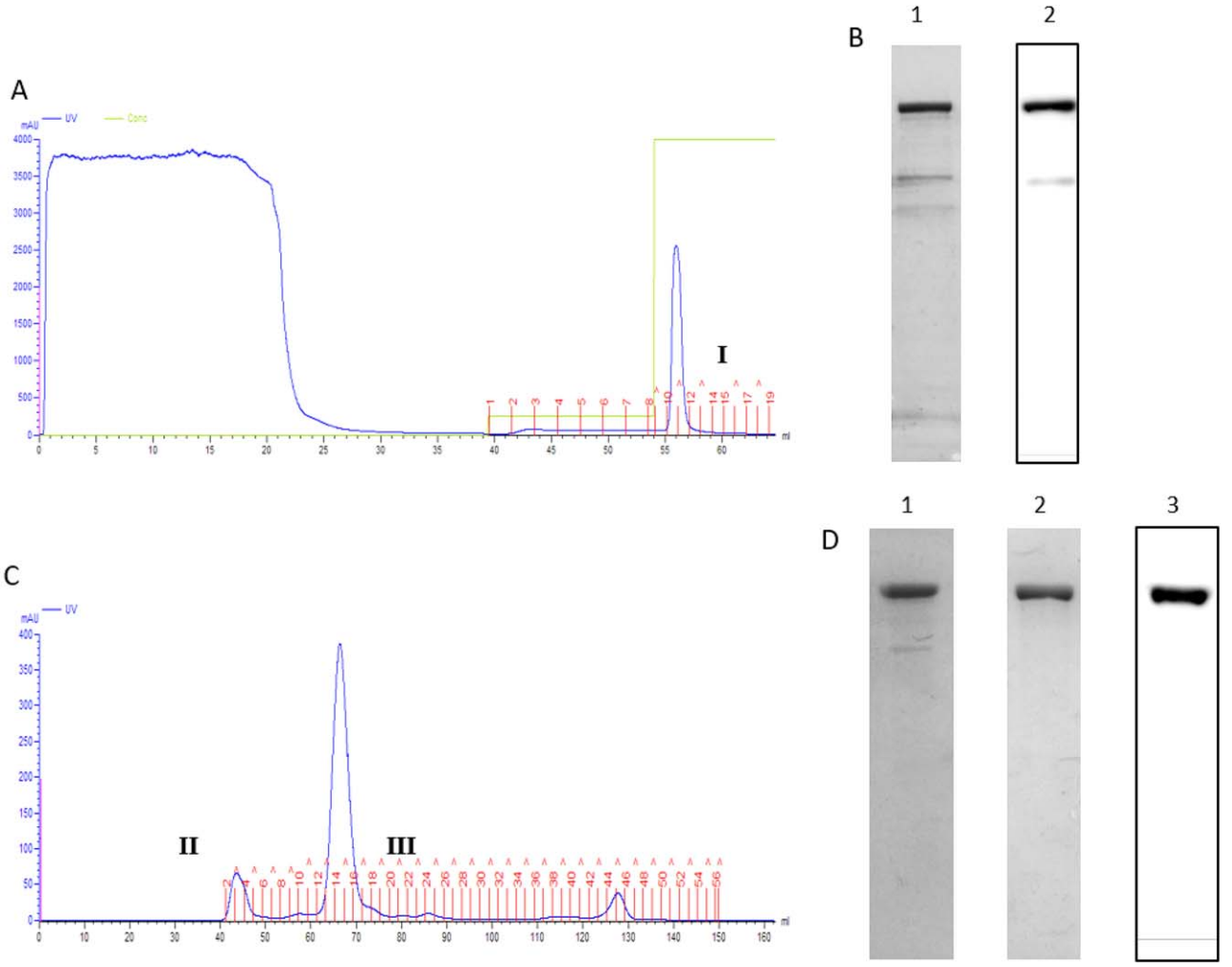

Figure 2. Two-steps purification of CNF1-H8. (A) Typical chromatogram of IMAC separation (HisTrap HP) of *E. coli* soluble cellular extracts after CNF1-H8 production. After flow-through discard, a washing step with mild imidazole concentration was followed by elution with 500 mM imidazole which led to the release of the target protein (Peak I). (B) Analysis of the eluted and pooled fractions after IMAC (Peak I). 1, SDS-PAGE; 2, Western blot. (C) The chromatogram of the second step purification of CNF1-H8 by size exclusion chromatography (Superdex 200 pg) displays two characteristic peaks, peak II representative of proteins released in the void volume of the column and peak III included in the Ve 55–65 mL range. (D) Analysis of the eluted and pooled fractions corresponding to Peaks II and III of the size exclusion chromatography. 1, SDS-PAGE related to Peak II; 2, SDS-PAGE of pure CNF1-H8 collected at the end of the purification procedure (Peak III); 3, pure CNF1 analyzed by Western blot (Peak III). 500 ng of total protein content was loaded in each lane of panels B and D.

for a fine biophysical characterization.[27,34,35] With the aim to overcome this limitation, we used an affinity chromatography to capture most of the recombinant protein followed by a size exclusion chromatography to increase the overall quality of the collected fractions (Figure 2). In detail, the clarified lysate harvested after cells disruption was applied to immobilized metal affinity chromatography (IMAC) using a 1 mL HisTrap HP column. The bound proteins were washed and eluted with IMAC Wash buffer and IMAC Elution buffer, respectively. The eluted fractions (peak I in Figure 2A) were pooled and analyzed by both SDS-PAGE (lane 1 in Figure 2B) and Western blot (lane 2 in Figure 2B). The densitometric analysis of the Coomassie stained denaturant gel revealed a 75% purity of the target protein, while the Western blot showed that at least one main contaminant in the sample was a degraded form of CNF1-H8, as it was detected by NG8 Mab (the same major proteolyzed fragment shown in Figure 1B). To eliminate the remaining contaminants, a size exclusion chromatography (Superdex 200 pg) was performed on partially purified CNF1-H8. The chromatogram presented two characteristic peaks, peak II eluted near the void limit of the column and peak III in the elution volume (Ve) range of 55–65 mL (Figure 2C). The SDS-PAGE related to peak II showed that a minor portion of the toxin was lost within the void volume of the column, possibly due to its partial aggregation (lane 1 in Figure 2D). Nevertheless, most of the CNF1-H8 eluted in peak III as shown by the SDS-PAGE and the Western blot analysis (lanes 2 and 3 in Figure 2D, respectively), which is compatible with the existence of a 116 kDa monomeric protein. Furthermore, the SDS-PAGE and Western blot performed on these collected fractions revealed an overall purity above 95% of the recombinant toxin in the final sample. In each lane of the Coomassie-stained gels and Western blot in Figures 2B,D, the amount of loaded proteins was normalized to 500 ng. Hence, the reader should refer to the chromatograms (Figures 2A,C) to have a concrete idea of the protein relative abundance among the different samples. The whole

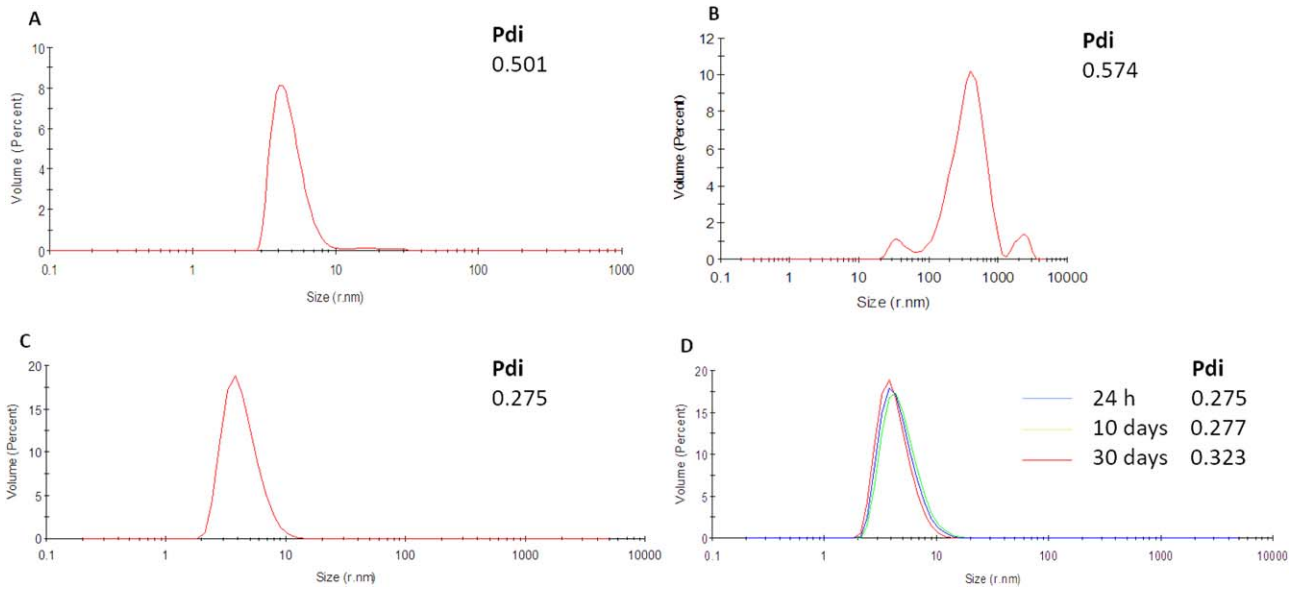

Figure 3. Size distribution of CNF1-H8 solutions throughout its purification. (A) DLS analysis of CNF1-H8 purified by IMAC (Peak I). (B) DLS analysis of aggregated CNF1-H8 collected during size exclusion chromatography (Peak II). (C) DLS analysis of pure CNF1-H8 harvested at the end of the purification (Peak III). (D) Definition of CNF1-H8 stability when stored at 4°C at a concentration of 10 mg/mL.

purification protocol led to the collection of 7–8 mg of recombinant protein per liter of culture in highly pure form. In our opinion, this is a significant result as this yield undoubtedly outperforms the purification of 1 mg of protein from a 4 L culture reported in a previous publication.[35] When the purification of CNF1-H8 was attempted on protein extracts collected after 20°C expression, we could not achieve the same level of purity depicted in Figure 2D after the two chromatographic steps, because the about 80 kDa degradation product (Figures 1B and 2B) was entirely co-eluted after IMAC and the Superdex 200 pg was not able to completely separate the two proteins when their relative abundances were similar (data not shown). A third chromatographic step was needed to reach the same purity level, but this led to a considerable loss in terms of yield (data not shown).

Evaluation of stability and dispersity of purified CNF1-H8

A detailed and extensive biophysical characterization of CNF1 recombinant protein requires that the final protein formulation is characterized not only by a high purity level, but also by acceptable homogeneity, solubility, and stability over time. To assess these features, DLS experiments were routinely performed to define the most suitable buffers to be used during the purification and storage of the protein.[47] Furthermore, this procedure was also useful to define the behavior of CNF1-H8 when preserved in concentrated form (up to 10 mg/mL) for long periods of time.[48] This feature is of pivotal importance as various structural and analytical techniques may require the use of highly concentrated and stable preparations. As consequence, the fulfillment of this goal could be crucial for a deeper structural characterization of CNF1.

First of all, DLS analysis was useful to define how the general quality of the protein preparations was increased in the two chromatographic steps. As shown in Figure 3A, CNF1-H8 collected after the IMAC was in a quite polydisperse state (Pdi 0.501). The following size exclusion chromatography led to the removal of complexed proteins characterized by a high hydrodynamic radius (Figure 3B) and to lower the Pdi of the protein solution to 0.275 (Figure 3C). This result confirms that peak II characteristic of the second step purification contained aggregated CNF1-H8 (Figure 2C). The almost monodisperse state of the final sample was retained up to 10 days when the protein was preserved in the storage buffer at 4°C at a concentration of 10 mg/mL. At increased times of incubation, a slight increase of dispersity was observed (Figure 3D). These data confirm that our simple purification protocol permitted to collect CNF1-H8 in a pure, concentrated and stable form.

Once the target protein had been purified, DLS analysis was used as described in *Materials and Methods* to study the protein behavior in different conditions mostly changing the buffers pH, ionic strength and testing additives known to stabilize proteins. Although CNF1-H8 seemed to be stable in a wide pH range (data not shown), the presence of salt and cosolvents (glycerol or glucose without a significant difference) exhibited a pivotal role in the preservation of the protein solubility and homogeneity. In fact, when CNF1-H8 was preserved in concentrated form (5–10 mg/mL) in 50 mM TrisHCl without additives and/or low NaCl concentration (up to 50 mM), it formed a turbid solution. This aggregation process was mainly affected by salt concentration, rather than other additives and this phenomenon was further confirmed by DLS experiments performed on the soluble fraction of these suspensions. As shown in Table 2, the co-presence of 200 mM NaCl and 15% v/v glycerol allowed to keep the Pdi as low as 0.3 at 4°C after 30 days storage. On the contrary, the single presence of glycerol with low NaCl content gave rise to a polydisperse suspension already after 24 h of incubation as indicated by a Pdi equal to 0.5. Finally, glycerol exhibited a minor still significant role in the preservation of CNF1-H8 stability, because the protein dissolved in high salt buffer without other additives experienced a faster Pdi increase over time than the samples in 200 mM NaCl, 15% v/v glycerol.

Thanks to these experiments, we could define that both a minimum NaCl concentration of 150 mM and the use of glycerol in the buffers could ameliorate the protein stability. For this reason, all the buffers used during the purification included these additives.

In vitro activity of CNF1-H8

Previous studies have demonstrated that the CNF1-catalyzed deamidation at position 63 in RhoA sequence can be monitored observing a typical electrophoretic shift in SDS-urea-PAGE.[6] This qualitative assay was exploited to confirm that the purified full-length protein preserved its catalytic activity. Following a previously reported protocol, RhoA was recombinantly produced and purified.[40] Then, RhoA was incubated with CNF1-H8 at 25°C in a 50:1 molar ratio for increasing times. The deamidated form of RhoA (RhoA E63) generated by CNF1-H8 action was expected to become progressively predominant over its unmodified form (RhoA Q63) and to show a delayed electrophoretic mobility. In accordance with the previous results,[33] RhoA deamidation was almost complete within 1 h as shown by the upward shift of RhoA characteristic band in an SDS-urea-PAGE experiment (Figure 4A). However, some kinetic differences were appreciated when CNF1-H8 was stored in different buffers. When the recombinant protein was preserved in high salt buffer with glycerol as a cosolvent, it provoked the conversion of about the 50% of the substrate in 10 min. Conversely, the absence of either NaCl or glycerol in the storage buffer caused a partial inactivation of the protein, as visible in the first and second rows of Figure 4A. These results together with the DLS analysis reported in Table 2 demonstrate that the removal of salt and glycerol from the preservation buffer provoked CNF1-H8 aggregation and that the partially soluble aggregates that are formed partially preserve their catalytic activity.

To further investigate the CNF1-H8 activity, experiments on HEp-2 cells, an epithelial cell line representing the reference cell line for CNF1 studies,[49] were performed. HEp-2 cells were treated with CNF1-H8 at different doses and when analyzed by means of a phase contrast microscope, the typical effect of the wild type CNF1 was observed, i.e., cell spreading, multinucleation and ruffling at cell border (Figure 4B). The observed effects were dose-dependent as visible in Figure 4C. To obtain an index of the CNF1-H8 activity, HEp-2 cells were treated with doses of CNF1-H8 starting from 10^{-7} M and decreasing until 10^{-16} M. The lower dose at which CNF1-H8 induced multinucleation in 50% of the cells was identified at 10^{-11} M. The induction of the typical phenotypic effects on cultured cells indicates that the purified toxin preserves its capability of being endocytosed, processed and released in the cell cytosol. Taken together, RhoA mobility assay and in vitro multinucleation assay confirmed that the protein purified with our protocol exerts its biological functions.

Table 2. Monitoring of CNF1-H8 Pdi in Different Storage Conditions

Tested additives	Pdi after 24 h	Pdi after 10 days	Pdi after 30 days
15% v/v glycerol, 200 mM NaCl	0.275	0.277	0.323
15% v/v glycerol	0.505	0.591	0.617
200 mM NaCl	0.316	0.339	0.430

DLS analysis was performed on the soluble fraction of CNF1-H8 dissolved in 50 mM TrisHCl in the presence of 200 mM NaCl, 15% v/v glycerol, or both at regular times.

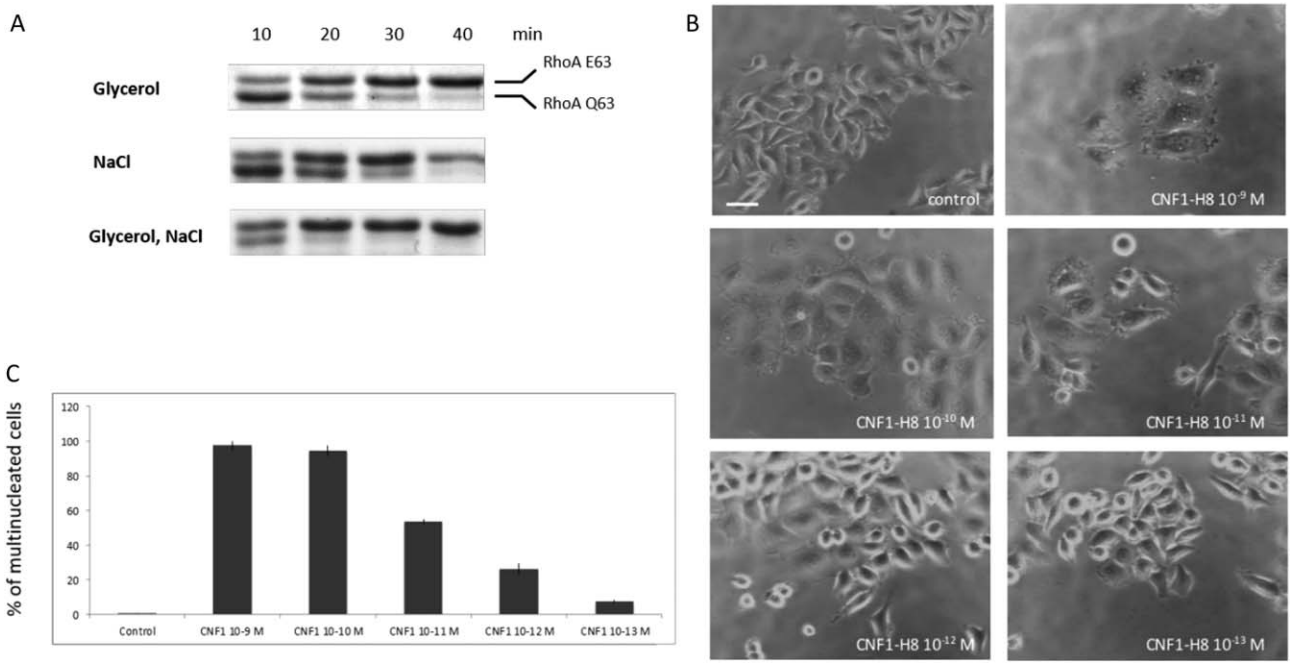

Figure 4. CNF1-H8 activity. (A) 200 nM CNF1-H8 and 10 μM RhoA were incubated at 25°C for the indicated times and an SDS-urea-PAGE was run to distinguish the substrate (RhoA Q63, lower band) from the product (RhoA E63, upper band) generated by the deamidation reaction. Before dilution in the Assay Buffer, CNF1-H8 was preserved in 50 mM TrisHCl buffer with 200 mM NaCl, or 15% v/v glycerol or both. (B) Phase contrast micrographs showing control Hep-2 cells and cells treated with different CNF1-H8 doses for 24 h. Bar represents 10 μm. (C) Percentage of multinucleated cells at different CNF1-H8 doses. Control: untreated cells.

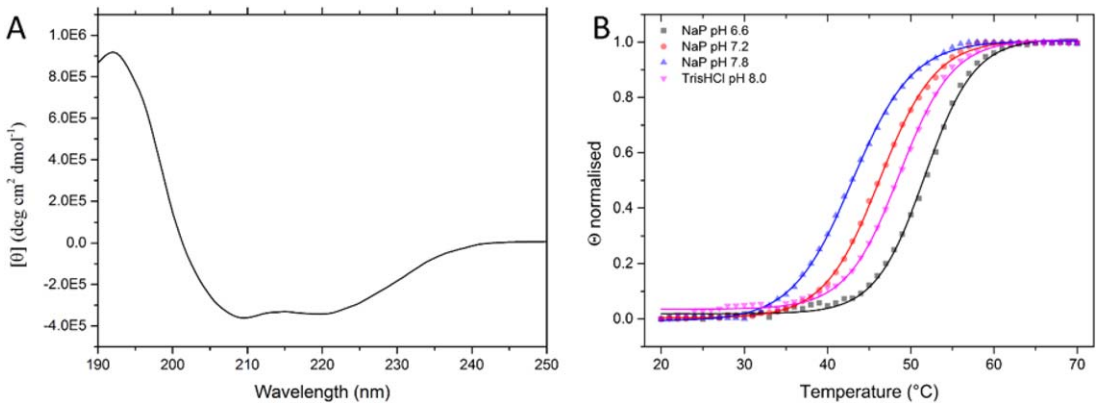

Figure 5. Circular dichroism analysis of CNF1-H8. (A) CD spectrum of CNF1-H8. (B) Normalized thermal denaturation curves of CNF1-H8 obtained by registering molar ellipticity at 209 nm. Spectra have been recorded by using 1 nM CNF1-H8 in 8 mM potassium phosphate buffers, 160 mM KF, 15% v/v glycerol. The pH 8.0 record has been obtained by using 8 mM TrisHCl. Temperature range 20–70°C, ramp 0.5°C min^{-1}, 60 s equilibration.

Structural studies of purified CNF1-H8

To date, only the catalytic domain of CNF1 (residues 720–1014) has been extensively characterized from a structural point of view. Its crystallographic structure had been solved at high resolution[32] and a number of spectroscopic and mutagenesis analyses had gone into its stability and kinetic features.[33] Nonetheless, literature comes with nearly no information concerning the remaining 70% of the protein. Here we propose an early structural characterization of the full-length protein, in terms of secondary structure composition and thermal stability.

The far-UV CD spectrum of the recombinant protein (Figure 5A), collected in 8 mM potassium phosphate buffer pH 7.2 and 160 mM KF at 20°C, is characterized by two slightly pronounced minima at 209 and 221 nm and a sharp maximum at 192 nm. Spectra deconvolution (see *Materials and Methods* section) gave rough, yet interesting clues about the extensively prevailing poorly structured regions (≈50%) over an estimate 35% of α-helices and 15% of β-strands. This had not come surprising, as broad flexible regions are like enough needed for the internalisation and processing stages within the host cell.[29,30]

The effect of pH on protein thermal stability has been studied by monitoring molar ellipticity at 209 nm in the temperature range 20–70°C (Figure 5B). The measurements have been performed by using the phosphate buffer whose pK_a has a small temperature dependence (dpK_a/dT = 0.0044 K^{-1}).[50] Interestingly, the pH sizably stabilizes CNF1-H8 as it moves down to acidic values: T_m goes from 43 ± 1°C at pH 7.8, to 45 ± 1°C at pH 7.2 and to 52 ± 1°C at pH 6.6 at last.

The thermal denaturation profile of the full-length protein has been also analyzed in conditions similar to those used in the study of the C-terminal catalytic domain:[33] 8 mM TrisHCl buffer pH 8.0 and 160 mM KF (purple line in Figure 5B). The melting temperature of the full-length protein (49 ± 1°C) is fairly close to that reported for the catalytic domain.[33] It is worth noting that the actual solution pH in these experimental conditions becomes a concern since TrisHCl buffer shows a not negligible dependence of pK_a on temperature (dpK_a/dT =- 0.028 K^{-1}).[50] Such a large pH variation does not allow a comparison between thermal stability data obtained in the two different buffers.

Conclusions

CNF1 is a bacterial toxin involved in the onset of some severe pathologies like meningitis and urinary tract infections.[1,2] Conversely, the same CNF1-induced mechanisms that seem to trigger the harmful effects of these diseases (i.e., Rho GTPases activation, wide cellular actin reorganization and alteration of mitochondria homeostasis) have been exploited to relieve the symptoms of other severe neurological diseases.[14–22] As a result, there is a great attention for an inclusive study of this protein at the molecular level to decipher both its action as a toxin and as a potential drug. At this point, we think that the level of detail devoted to the structural characterization of the catalytic domain of the protein should be extended to the full-length CNF1.

In this study, we propose a procedure for CNF1 production and purification aimed to minimize its instability and degradation issues and to collect it in a sufficiently pure, concentrated and homogeneous state. The protein, recombinantly expressed in *E. coli* BL21(DE3) as a his-tagged product (CNF1-H8), was purified using two chromatographic steps performed in native conditions, which permitted to collect up to 8 mg of pure and active protein per liter of culture, the highest yield reported for this protein so far. DLS analysis was used to optimize the buffer formulation to preserve CNF1-H8 in a highly concentrated, homogeneous and stable form. Such an approach allowed us to conduct the first general structural characterization of the whole CNF1 sequence, and it opens up interesting opportunity to perform a deep biochemical characterization, necessary to achieve a clear understanding of the CNF1 mechanism of action and its potential therapeutic use.

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1.4 Discussion

As reported in the previous section, the work carried out in the first year of this PhD program was useful to define a simple protocol for the recombinant production and purification of a sufficient amount of CNF1 for its following functional *in vivo* characterization. Although this characterization has never been carried out, it might be useful to define the critical issues that may arise during the development of a protein drug based on CNF1 toxin.

A. Fabbri and co-workers have generally suggested that the main obstacle in the use of this protein as a therapeutic involves its administration method (Fabbri, Travaglione and Fiorentini, 2010). As a matter of fact, all the case studies involving rodents as test subjects were performed using intracerebroventricular (icv) injections of CNF1. A systemic administration does not seem to be feasible because of the high molecular weight of the protein and its incapacity to cross the blood brain barrier (BBB). In my honest opinion, both the assertions are quite questionable.

First of all, the incapability of the protein to penetrate the BBB is not a confirmed datum. As mentioned in Section 1.1, CNF1 seems to be involved in meningitis and to actively contribute to bacterial invasion of the brain (Wang and Kim, 2013). The understanding of the entire process of infection is complicated by apparently contradicting results achieved by independent experiments which are probably the consequence of tissue-specific effects, the employment of different animal models and dosage (Boyer, 2016). Despite that, it is sure that CNF1 is capable of interacting with human brain microvascular endothelial cells (HBMECs), invade them and facilitating bacterial penetration across the BBB (Khan *et al.*, 2002, 2003; Chung *et al.*, 2003). It is not clear if the toxin remains trapped inside the endothelial cells, but it is quite obvious that it highjacks them to facilitate the transport of materials (even as big as bacteria) from the luminal to the abluminal side of brain capillaries (Wang and Kim, 2013). Intriguingly, in a recent review C. Fiorentini's group asserted again that CNF1 is not capable of crossing the BBB, but also that the recombinant CNF1-H8 purified by us can do that (Maroccia *et al.*, 2018). Unfortunately, they were not so kind to share their results with their collaborators and we can only blindly accept the fact that the protein may autonomously enter the brain parenchyma and it is very unlikely that the observed phenomenon may be due to the C-terminal oligohistidine tag.

However, regardless of its supposed intrinsic BBB crossing abilities, CNF1 cannot be intravenously injected as it is. Probably, a systemic administration is not pursuable at all with the current technologies. The case of CNF1 is particularly problematic both because the target is difficult to reach and because the supposed drug is a toxin. Hence, the drug development should aim to reach the highest accumulation in the brain and to avoid off-target dispersion. To do so, the interaction between the protein and its receptors has to be abolished, as they are expressed in several tissues, including blood cells (Parsons *et al.*, 1995; Rahuel *et al.*, 1996; Ardini *et al.*, 1998; Kikkawa and Miner, 2005). Therefore, an intuitive strategy would consist in the coupling of the C-terminal catalytic domain of the protein with an addressing molecule for the BBB crossing. Nevertheless, even this approach does not seem convincing, because the current strategies used for delivering proteins towards the brain parenchyma are neither specific nor efficient enough. Regardless of the used carrier (antibodies, cell penetrating peptides, decorated vesicles and nanoparticles), off-target accumulation is always a common trait and the scientific community considers a brain uptake ≥ 1% of the injected dose as a positive result (Boado *et al.*, 2007; Van Rooy *et al.*, 2011; Zhou *et al.*, 2011). If such an objective might be considered satisfying for those drugs whose dispersion in the body is not detrimental, the same cannot be

accepted in the case of a toxin. As reported in Section 1.1, CNF1 alters many biochemical processes inside a cell and the outcome is very dependent on the tissue, the dosage and the time of observation (Lemonnier, Landraud and Lemichez, 2007). This is mainly due to the fact that depending on the cellular context, the kinetics of activation, degradation and re-synthesis of Rho GTPases is different and forecasting which G protein lasts for a longer period in each environment is rather complex. This odd situation can be easily appreciated by comparing Figure 1.1 and Figure 1.2, where the general effects of CNF1 action are schematized in endothelial and cerebral tissues. As the reader can see, in many cases the cellular responses triggered by CNF1 are totally opposite: in one case the toxin causes the activation of the inflammosome (Figure 1.1), while in the other a reduction of ROS genesis (Figure 1.2); in a normal context the activation of phosphorylation cascades has a prosurvival effect on the intoxicated cells, inducing their proliferation, while in glioma the same processes induce cell death. In conclusion, in my honest opinion a systemic administration of CNF1 or its derivatives is not feasible with the current widespread methods because of the lack of specificity and efficacy.

Paradoxically, icv injection may represent the safest way to deliver CNF1, because it would consist in a topic treatment preventing an uncontrolled spreading of the toxin in the human body. Nevertheless, also this approach presents some obscure points. First of all, Malchiodi-Albedi clearly demonstrated that also in the brain district CNF1 provokes either positive or negative effects depending on the type of cell it comes in contact with (Malchiodi-Albedi *et al.*, 2012), as it seems to be deleterious for neurons and beneficial for astrocytes. The targeting of a precise cell type rather than another might be very difficult, considering that astrocytes and neurons are in close communication. Moreover, CNF1 exploits extracellular vesicles for cell-to-cell propagation and this can constitute a further cause of undesired uncontrolled diffusion of the protein in different brain districts (Fabbri *et al.*, 2015). Furthermore, no toxicity studies have ever been performed even in the CNS. For example, what happens at the BBB level when CNF1 is icv injected? Is it still capable of contacting HBMECs from the abluminal side? CNF1 receptors are expressed in endothelial cells mainly at the basolateral side and are theoretically available for the interaction with their ligands (Kim, Chung and Kim, 2005). This suggests that CNF1 could still possibly invade the BBB and induce a meningitis-like leakiness. Finally, the beneficial effects exerted by the toxin in the CNS were observed also in healthy mice, indicating that it induces a wide alteration of the brain functioning even in normal conditions (Diana *et al.*, 2007).

All these considerations suggest that the development of a therapeutic drug based on CNF1 may encounter several obstacles. First of all, a systemic administration of the toxin does not seem convincing because of the several issues concerning the biodistribution and safety. Furthermore, I personally believe that it is quite unlikely that CNF1 might be used for therapeutic purposes even when directly injected in the brain, because of the dramatic and variable alterations that are induced in the different CNS districts both in pathological and normal conditions. One has also to consider that deamidation of proteins, like the one catalyzed by CNF1, is very often linked to processes of aging and carcinogenesis (Deverman *et al.*, 2002; Robinson, 2002) and that chronic infections of CNF1-producing strains have been connected with cancer (Fabbri *et al.*, 2013). Considering that this protein only relieves the symptoms of CNS disorders, it is likely that cumulative injections are needed to preserve the therapeutic effect and such a situation might recapitulate a condition very similar to a chronic infection.

The unpredictability of a therapeutic treatment involving CNF1 mainly descends from the targets of this toxin. Rho GTPases are at the highest level of so many regulatory pathways involving gene expression, metabolism, cellular shape, movement and proliferation (Etienne-Manneville and Hall, 2002) that achieving only the desired effect without perturbing all the other processes is a though task. That is why I think that the usefulness of this protein in health sciences resides more in its employment as a tool for studying pathologies involving the abovementioned processes, rather than to treat them.

1.5 References

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Chapter 2: Recombinant production of a protein involved in human cerebral development in *Pseudoalteromonas haloplanktis* TAC125

Note:
As a part of this project was developed with the funds of Amicus Therapeutics Inc., the results achieved in this field are regulated by a secrecy agreement. For this reason, the real name of the produced protein has been conveniently replaced with the expression “Variant B” in this text. Furthermore, only the essential information about the function and the chemical properties of the protein is reported.

2.1 Introduction

2.1.1 Variant B: a paramount protein in brain development

Variant B is produced in mammals in different tissues and it has been reported to be essential for a normal development of the CNS. Mutations in its gene induce pathologic phenotypes, whose gravity depends on the extent of the impairment of Variant B normal functioning. All the molecular processes this protein is involved in are still not completely understood, but we know that it exerts its action both in the cell nucleus, in the cytoplasm and at the level of the cytoplasmic membrane. Variant B responds to extracellular stimuli and is capable of transducing these signals both at the genetic level and influencing important biochemical regulatory pathways.
Such a heterogeneity in terms of cellular localization and function is a common feature of intrinsically disordered proteins (IDP) (Uversky, 2016), which are characterized by an uncommon flexibility allowing them to meet several different molecular partners. Variant B seems to respond to these structural criteria, as only about one third of the protein seems to be precisely structured, while the remaining part is predicted to be flexible. This structural peculiarity makes Variant B a difficult-to-express protein in the most conventional hosts. It tends, in fact, to be either heavily proteolyzed or to accumulate in insoluble aggregates. Eukaryotic expression platforms allowed to collect some active protein, but the overall yields are unsatisfying for any application.
Hence, biotechnological companies have started to probe alternative and more exotic recombinant systems in the hope of finding a new cellular context for the proper structuration and preservation of Variant B.

2.1.2 *Pseudoalteromonas haloplanktis* TAC125: an exotic bacterium for the recombinant production of human proteins

Pseudoalteromonas haloplanktis TAC125 (*Ph*TAC125) may represent a good candidate for the production of Variant B, because of its favorable physiological features including fast growth at low temperatures and efficient protein synthesis. Furthermore, a series of proof-of-concept studies have already demonstrated its suitability for the recombinant expression of human proteins.
*Ph*TAC125 is an Antarctic marine γ-proteobacterium (Birolo *et al.*, 2000) and is the first polar bacterium whose genome has been fully sequenced and carefully annotated (Médigue *et al.*, 2005). Table 2.1 shows the main features of *Ph*TAC125 genome. As in most of the marine γ-proteobacteria, it contains two differently sized chromosomes which possess different mechanisms of replication. Chromosome B (chrB) resembles a megaplasmid, as it presents a RepA-dependent unidirectional replication and about 20% of its predicted coding sequences (CDSs) are homologues of plasmid-encoded genes. Nevertheless, during evolution chrB has become a stable genetic element in *Ph*TAC125 for the preservation of essential genes involved in the central metabolism (Médigue *et al.*, 2005). One of the most interesting features of *Ph*TAC125 genome

involves its translational capacity. The existence of 9 rRNA clusters and 106 tRNA genes should guarantee a uniform translation rate over time avoiding ribosome stalling processes even in very demanding situations. This might justify the relatively fast growth showed by the bacterium in a wide range of temperatures (-2.5 – 30 °C) in differently defined growth media (Sannino *et al.*, 2017). From a metabolic point of view, the most striking trait of *Ph*TAC125 is the absence of a phosphoenolpyruvate phoshphotransferase transport system and of a glycolytic pathway. Genomic analysis and proteomics studies (Wilmes *et al.*, 2011) indicated that this bacterium preferentially uses amino acids as carbon and nitrogen sources. In fact, it can rapidly grow in rich media containing either peptone or casaminoacids (Papa *et al.*, 2007; Wilmes *et al.*, 2010) or in more defined broths containing few amino acids (Giuliani *et al.*, 2011). In their genetic analysis Médigue et al suggested that gluconate could be a preferred carbon source metabolized through the Entner-Doudoroff pathway (Médigue *et al.*, 2005), while Wilmes and co-workes showed that glutamate is the amino acid that mostly contributes to biomass formation in an amino acids mixture (Wilmes *et al.*, 2010). Recently, Sannino *et al.* took inspiration from these two observations and mixed 10 g of gluconate with 10 g of glutamate in 1 L of a mineral salt base so to formulate the so-called GG medium that allows discrete growth performances at various temperatures (Sannino *et al.*, 2017). Nevertheless, an efficient medium allowing a fast single substrate-limited growth has not been established yet. This deficiency limits the process optimization for a controlled bacterial growth, like the establishment of a fed-batch operation, because the behavior of the bacterium is less predictable in a mixed substrate culture (Kovarova-Kovar and Egli, 1998). In fact, the lack of a canonical cAMP-CAP catabolite repression system in *Ph*TAC125 (Médigue *et al.*, 2005) does not automatically mean that there is not any metabolic cross-regulation. For example, amino acids are consumed with precise hierarchies in *Ph*TAC125 (Wilmes *et al.*, 2010) or can interfere with the uptake of other nutrients, like malate (Giuliani *et al.*, 2011). Even in the GG medium we observed that only gluconate is assimilated during the first hours of the growth and then, after a latency, also glutamate is started to be consumed, giving rise to a sort of diauxic growth (personal communication). Actually, the GG medium as formulated in 2017 (Sannino *et al.*, 2017) seems to be unbalanced because of the overabundance of carbon sources in comparison with the mineral salts, so that a batch carbon-limited growth is not achievable. We recently overcame this issue by drastically changing the mineral salt base composition and by significantly lowering glutamate and gluconate initial concentrations. This re-arranged formulation might constitute a good starting point for future process engineering studies.

Table 2.1. Genetic elements of *Ph*TAC125 genome and their features.

DNA molecule	Size (bp)	N. of predicted CDSs	Replication	Reference
Chromosome A	3214944	2942	DnaA	Médigue *et al.*, 2005
Chromosome B	635328	546	RepA	Médigue *et al.*, 2005
Plasmid pMtBL	4086	2	Unknown	Tutino *et al.*, 2001 Dziewit *et al.*, 2014
Plasmid *Ph*TAC125 2	Unknown	Unknown	Rep-3	Unpublished

*Ph*TAC125 possesses at least other two genetic elements besides its chromosomes (Table 2.1). The first is the plasmid pMtBL (Tutino *et al.*, 2001), a small replicon of about 4 kb which does not seem to carry any vital CDS for the bacterial sustainment.

Although its replication origin has been isolated by restriction analysis, nothing is known about its mechanism. A general analysis of the sequence just indicates the lack of a typical GC skew, suggesting a unidirectional replication (AJ224742, NCBI), but any crucial element for DNA replication has never been identified. Very recently we found evidence of the existence of a second plasmid in *Ph*TAC125. A re-analysis of the DNA sequences belonging to a library built from *Ph*TAC125 genome (Duilio *et al.*, 2004) indicated that the sequence called P15 (AJ557248.1, NCBI) is not present in the bacterial genome. A following BLASTN search revealed an almost 100% identity of P15 with a portion of a *Pseudoalteromonas* plasmid and in particular with the 3' end of a predicted CDS (ASM56327.1, NCBI). The prediction of conserved domains on the translated hypothetical protein suggested that it belongs to a Rep-3 family involved in plasmid replication. Recently, M. Fondi and co-workers of the University of Florence re-sequenced *Ph*TAC125 whole genome and they also found new DNA sequences diverse from the ones obtained in 2005 (Médigue *et al.*, 2005) including the one of P15. This suggests that P15 is not a result of a contamination during the building of the library in 2004 and probably belongs to an endogenous plasmid (Duilio *et al.*, 2004). The assemble of the obtained contigs will be of pivotal importance for understanding the entire mobilome of this bacterium with useful outcomes both for basic research and for its exploitation in recombinant proteins productions.
However, the discovery of pMtBL in 2001 opened the possibility of using this psychrophilic microorganism as a host for recombinant processes. The fusion of DNA sequences derived from *E. coli* vectors, with psychrophilic bricks allowed the development of a shuttle vector whose manipulation and amplification are easily achievable in conventional *E. coli* strains, while recombinant expression is pursued in the marine bacterium (Tutino *et al.*, 2001). As shown in Figure 2.1, a typical *Ph*TAC125 expression vector (pX) presents a selection marker gene and a mesophilic origin of replication (*OriC*) derived from pUC18 vector, the psychrophilic origin of replication (*OriR*) from pMtBL, a conjugational transfer origin *(OriT*) derived from pJB3 vector for interspecific conjugation, and a psychrophilic transcriptional terminator (*PhaspC term*) (Birolo *et al.*, 2000) just downstream of the multiple cloning site (MCS).

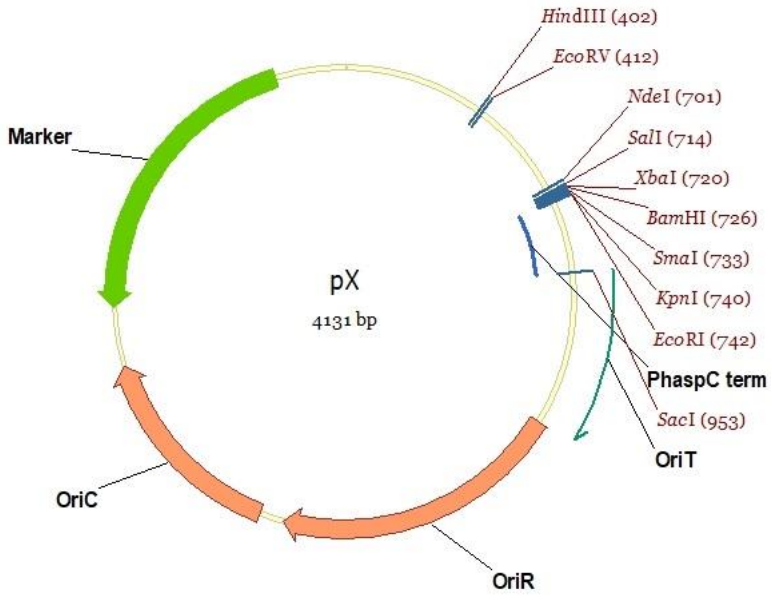

Figure 2.1 The shuttle vector backbone. *OriC*, pUC18-derived origin of replication; *OriR*, pMtBL-derived autonomous replication sequence; Marker, resistance to antibiotics; *OriT*, conjugational DNA transfer origin; *PhaspC term*, transcriptional terminator.

The MCS has been used to introduce various expression cassettes for recombinant proteins production. As mentioned before, constitutive expression of genes of interest has been made possible by the development of a library of supposedly constitutive promoters characterized by variable strengths (Duilio *et al.*, 2004). Following studies have led to the design of two inducible expression vectors using L-malate (Papa *et al.*, 2007) and D-galactose (Sannino *et al.*, 2017) as inducer molecules so to allow a more versatile and tunable recombinant expression. Finally, a fruitful secretion platform has been set up by both implementing translocation peptides for the periplasmic localization (Vigentini *et al.*, 2006) and for the extracellular addressing (Cusano, Parrilli, Marino, *et al.*, 2006). The biotechnological potential of *Ph*TAC125 has been increased by the demonstration that conventional techniques for genomic DNA manipulation can be employed (Giuliani *et al.*, 2012) also in this bacterium. This approach has been efficiently used to reduce the extracellular proteolytic activity of *Ph*TAC125 with no drawbacks for the microorganism physiology (Parrilli *et al.*, 2008). The abovementioned genetic toolkit coupled to the biotechnologically relevant physiological features of *Ph*TAC125 has been exploited by ML Tutino and coworkers for the effective production of difficult-to-express eukaryotic proteins in proof-of-concept studies, the human nerve growth factor (Vigentini *et al.*, 2006), the human alpha-galactosidase (Unzueta *et al.*, 2015) and antibody fragments being the best examples (Giuliani *et al.*, 2014).

2.2 Aim of the study

This study was commissioned by Amicus Therapeutics Inc. Its main aim was the full-length and soluble production of human Variant B in *Ph*TAC125. In perspective this study will be useful to define the suitability of the psychrophilic expression platform for the large scale recombinant production of the protein to be used both for basic research and for therapeutic purposes. The results achieved in this filed will not be showed for three years because of a secrecy agreement.

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Appendix

Oral communication

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Microbial Cell Factories

RESEARCH

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Getting value from the waste: recombinant production of a sweet protein by *Lactococcus lactis* grown on cheese whey

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Abstract

Background: Recent biotechnological advancements have allowed for the adoption of *Lactococcus lactis*, a typical component of starter cultures used in food industry, as the host for the production of food-grade recombinant targets. Among several advantages, *L. lactis* has the important feature of growing on lactose, the main carbohydrate in milk and a majoritarian component of dairy wastes, such as cheese whey.

Results: We have used recombinant *L. lactis* NZ9000 carrying the nisin inducible pNZ8148 vector to produce MNEI, a small sweet protein derived from monellin, with potential for food industry applications as a high intensity sweetener. We have been able to sustain this production using a medium based on the cheese whey from the production of ricotta cheese, with minimal pre-treatment of the waste. As a proof of concept, we have also tested these conditions for the production of MMP-9, a protein that had been previously successfully obtained from *L. lactis* cultures in standard growth conditions.

Conclusions: Other than presenting a new system for the recombinant production of MNEI, more compliant with its potential applications in food industry, our results introduce a strategy to valorize dairy effluents through the synthesis of high added value recombinant proteins. Interestingly, the possibility of using this whey-derived medium relied greatly on the choice of the appropriate codon usage for the target gene. In fact, when a gene optimized for *L. lactis* was used, the production of MNEI proceeded with good yields. On the other hand, when an *E. coli* optimized gene was employed, protein synthesis was greatly reduced, to the point of being completely abated in the cheese whey-based medium. The production of MMP-9 was comparable to what observed in the reference conditions.

Keywords: *Lactococcus lactis*, MNEI, Nisin controlled expression system, Cheese whey, GRAS, Bioconversions, Recombinant proteins

Background

Lactic acid bacteria (LAB) are traditional components of starter preparations and have been used for centuries in the manufacturing of fermented food [1]. More recently, they have attracted much attention for their biotechnological potential, finding use in a variety of applications. *Lactococcus lactis* (*L. lactis*), a prototypical member of this family, is a gram-positive, non-sporulating, facultative anaerobic bacterium and a common gut colonizer, which has been used for centuries by the cheese-making industry, thus receiving the Generally Recognized As Safe (GRAS) status [2, 3]. Outside food industry, *L. lactis* has been successfully exploited for its metabolic machinery, to produce and accumulate high value chemicals, such as ethanol, L-lactate, diacetyl, acetaldehyde, but also L-alanine, mannitol and other sweeteners and group B vitamins [3, 4]. Much of the biotechnological advances that have taken place for *L. lactis* own to the achievement of complete genome sequencing of a few strains [5, 6], which has allowed for metabolic

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engineering manipulations, aimed at favoring the production of specific metabolites of industrial interest [4, 7–10]. In this respect, it is noteworthy the production of bacteriocins, antimicrobial peptides with a number of applications, ranging from food-preservation to anti-biofilm activity in clinical set-ups [11–13].

Together with genome sequencing, the discovery and development of both constitutive and inducible expression systems for *L. lactis* has favored its development as a cell factory for the production of recombinant proteins [2, 14, 15]. Being a food-grade microorganism, devoid of endotoxins, *L. lactis* is very convenient for the heterologous production, both intracellular and secreted, of a number of therapeutics and vaccines, recently reviewed by Song et al. in [3].

The greatest boost in the use of *L. lactis* for the production of heterologous protein has been the development of the nisin controlled gene expression (NICE) system [16], which has been used in combination with the nisin-negative NZ9000 strain and its derivatives for the production of numerous recombinant protein targets [17].

In parallel with their use as molecular biology tools, *L. lactis* and other LAB have found space in the field of bioconversions, where their ability to digest the components of industrial effluents has been coupled to the production of added value chemicals [18–25]. In the case of LAB, their ability to digest lactose has been functional to the possibility of using them for the valorization of dairy wastes. Cheese whey (CW), the serum portion of milk that survives the process of cheese making, is in fact one of the most polluting by-products of dairy industry and its disposal imposes a considerable economic burden on cheese producers, due to its high biological and chemical oxygen demand [26]. The composition of CW varies according to its origin, but, in general, it is a nutritious mixture, containing roughly half the solids of raw milk. In particular, most of the lactose (that survives protein agglutination), roughly 20% of the original milk proteins, some vitamins and minerals are still present in CW, which is in fact often used as feeding for livestock [27–29]. In addition to its use "as is", strategies for the valorization of CW have been proposed, often employing microbes to convert it in biomass or added value compounds, like ethanol, organic acids or bacteriocins [18, 20, 28, 30–32]. Nonetheless, to our knowledge, this material has never been used to sustain the production of high value recombinant proteins.

We here describe the use of the CW resulting from the making of mozzarella and ricotta cheese, to sustain the growth of *L. lactis* NZ9000 and the heterologous production of the small (~12 kDa), globular protein MNEI. MNEI is a single chain derivative of the plant (*Dioscoreophyllum cumminsii*) protein monellin [33, 34] and has received much attention for its high intensity sweetness. Due to this potential industrial interest, several biotechnological strategies have been devised to produce it, exploiting a variety of host systems [35, 36]. MNEI is one of the best characterized members of the sweet proteins family and it has been object of protein engineering to improve its taste profile and physicochemical characteristics, trying to meet the needs of the food and beverage industry [37–43]. In light of its potential applications in food preparations, the optimization of a food-grade production system becomes particularly important. The possibility of coupling such system to the valorization of a largely available industrial by-product makes our strategy particularly appealing. Moreover, we demonstrate that these growth conditions can be successfully used to produce other recombinant proteins in *L. lactis* strains. In particular, we show that the expression levels of the previously characterized protein MMP-9 in *L. lactis* [44, 45] are comparable in the CW-based medium and in the reference conditions in rich medium.

Results

Expression of MNEI in *L. lactis* NZ9000

The synthetic genes coding for MNEI with optimized codon usage for *E. coli* (*MNEI-ec*) and *L. lactis* (*MNEI-ll*) were cloned into the pNZ8148 vector. This was motivated by the fact that previous studies aimed at the production of brazzein, another sweet protein, in *L. lactis* had shown the counterintuitive result of greater production yields when employing an *E. coli* optimized gene [46–48]. In our case, the two synthetic genes differed for their GC content (49% and 37% for *MNEI-ec* and *MNEI-ll*, respectively) and their sequence alignment is reported in Additional file 1: Figure S1. Unlike the previous results for brazzein, when *L. lactis* NZ9000 was transformed with the vector containing the *MNEI-ec* gene, negligible protein production was observed (Additional file 2: Figure S2). Conversely, the system carrying the pNZ8148-*MNEI-ll* vector proved to be quite efficient: Fig. 1 shows the Coomassie stained SDS-PAGE and the western blot of 10 µg of total protein extracts from recombinant *L. lactis* producing MNEI. Densitometric quantification of the blot provided an estimate of the yield ~140 ng MNEI in 10 µg of total protein extract, corresponding to ~0.40 mg of protein per liter of culture medium, which remains quite stable throughout the fermentation, based on the intensity of the bands corresponding to 2, 4 and 16 h post-induction in comparison to purified protein samples.

Growth of *L. lactis* NZ9000 on cheese whey based media

The above experiments were all carried out in the conventional growth conditions for *L. lactis*, namely static

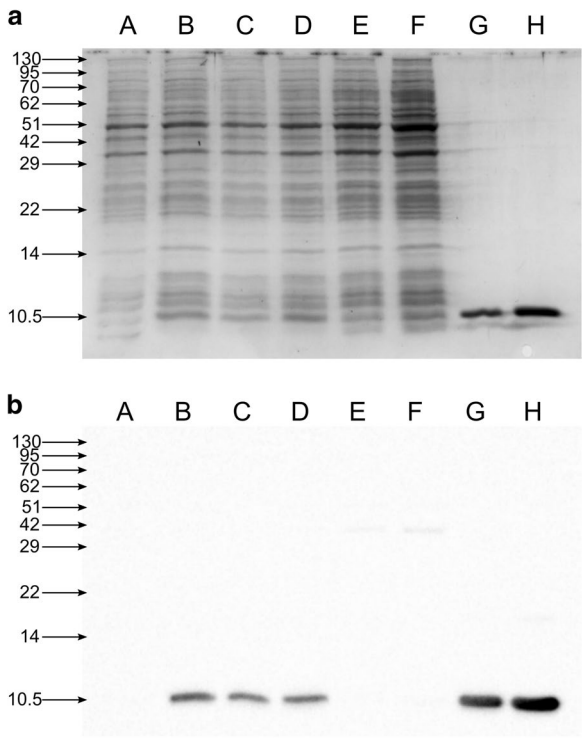

Fig. 1 Production of recombinant MNEI in G-M17 medium. Coomassie-stained SDS-PAGE (**a**) and western blot (**b**) of the total protein extract (10 µg) from *L. lactis* NZ9000 carrying the pNZ8148-*MNEI-II* vector (A–D) or the empty pNZ8148 vector (E–F). A: no induction; B: 2 h post-induction; C: 4 h post induction; D: 16 h post induction; E: no induction; F: 4 h post induction; G: MNEI standard, 200 ng; H: MNEI standard, 500 ng

cultures in M17 rich medium supplemented with 0.5% glucose (G-M17) at 30 °C. We then moved to comparing the microbial growth on CW. We used the CW obtained as by-product of the manufacturing of ricotta, a typical fresh cheese from Campania, Italy. Ricotta is produced by heating at 90 °C the whey resulting from the production of "Mozzarella di Bufala Campana" and collecting the flocculating suspension. The residual watery portion constitutes the CW employed in this study. Upon delivery to our lab, all CW samples contained variable amounts of particulate in suspension, formed during the cooling down of the exhausted whey. Several treatments have been proposed as preliminary workups when CW is used as a raw material for biotechnological applications, and these range from total deproteinization of CW, to treatment with proteolytic enzymes, to supplementation with other nutrients such as peptone and yeast extract [20, 21]. Since we were trying to set up a scalable protocol, ideally suitable to work on the large amounts of CW produced by a dairy industry, we decided to limit the steps prior to inoculation

Table 1 Mean composition and standard deviation of the cheese whey (CW) at reception and after sterilization/clarification (ac-CW)

	CW	ac-CW
Carbohydrates (mg/mL)	27 ± 2	40 ± 5
Proteins (mg/mL)	2.4 ± 0.3	2.3 ± 0.2
Lipids (mg/mL)	12 ± 5	7 ± 3
pH	6.1 ± 0.1	6.17 ± 0.04

of *L. lactis* to pH adjustment to 6.8, sterilization by autoclave and clarification by centrifugation of CW (ac-CW). The average nutrients content of CW and ac-CW before bacterial culture is provided in Table 1. The counterintuitive increase in carbohydrate content upon sterilization might result from partial hydrolysis of lactose, the main sugar of milk, to glucose and galactose. This would provide a higher response to the phenol assay used to quantify reducing carbohydrates. Despite pH adjustment had been performed, the lack of buffering capacity of untreated CW resulted in a drop in pH after sterilization, to ~ 6.2. Nonetheless, given the tolerance of LAB for acidic pHs, no further adjustment was performed. The medium was also supplemented with a catalytic amount (0.05%) of yeast extract as a source of vitamins and cofactors, to improve the utilization of the nutrients, still abundant in ac-CW.

Explorative experiments showed that the growth on the CW-based medium was accompanied by a marked decrease in pH, due to the production of organic acids, paralleling what observed during the growth in G-M17 medium (Additional file 3: Figure S3). Therefore, since the proteins in CW had not been hydrolyzed, the growth in this medium was accompanied by additional protein precipitation, making it impossible to monitor biomass accumulation with turbidimetric methods (i.e. by measuring the OD600). Thus, all the growth curves were constructed measuring the CFUs/mL at different time points. Figure 2 shows the comparison of the growth curves, while Table 2 summarizes the growth parameters, for *L. lactis* NZ9000 in the standardized G-M17 medium and in the ac-CW-based medium. The marked difference in biomass at the end of the growth (2.14×10^8 vs 1.04×10^9 CFU/mL in ac-CW medium and G-M17, respectively) is likely the result of the significant protein deficiency of the ac-CW-based medium compared to G-M17 (2.3 vs 15.0 g/L, Additional file 4: Table S1) and of an unbalanced carbohydrates:proteins ratio (40:2.3 vs 10:15). Nonetheless, despite these suboptimal conditions, *L. lactis* NZ9000 could thrive in the CW-derived medium.

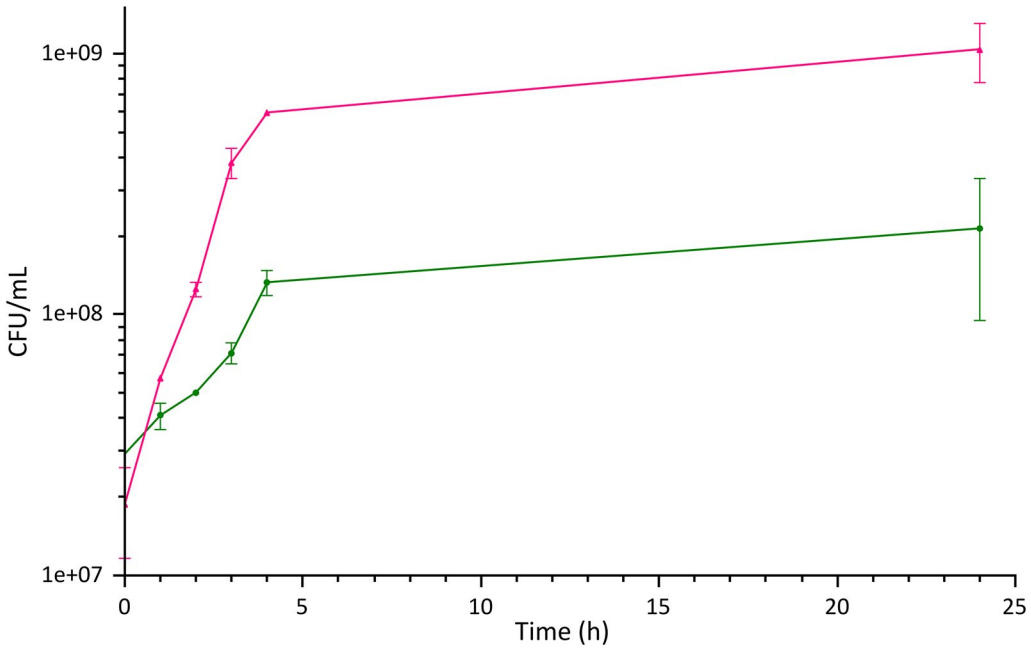

Fig. 2 Growth curves of *L. lactis* NZ9000 in different media. The figure shows a comparison of the growth curve of *L. lactis* NZ9000 in the reference rich medium G-M17 (red curve) and on ac-CW supplemented with 0.05% yeast extract as a source of cofactors and vitamins (green curve). The final biomass in the two conditions differs by one order of magnitude

Table 2 Comparison of the growth parameters for *L. lactis* NZ9000 in G-M17 medium and ac-CW + 0.05% yeast extract

	Growth rate μmax (h^{-1})	Doubling time td (h)
G-M17	1.1 ± 0.2	0.6 ± 0.1
Ac-CW + 0.05% yeast extract	0.6 ± 0.1	1.2 ± 0.1

Expression of recombinant MNEI on CW-based medium

Once we had ascertained the capacity of *L. lactis* to grow on the ac-CW-based medium, we checked if these conditions could also sustain the expression of MNEI in the recombinant strain carrying the pNZ8148-*MNEI-ll* or pNZ8148-*MNEI-ec* vector. Both strains showed comparable growth profiles to the wild type strain (not shown); therefore, in all subsequent experiments, protein synthesis was induced after 2 h, corresponding to the mid-exponential phase, with 10 ng/mL nisin and checked at different time points post-induction by Western Blot. Figure 3 shows the Coomassie-stained SDS-PAGE (A) and Western blot (B) of the total protein extracts at different times post induction. Despite the discussed reduction in biomass accumulation compared to the standard G-M17 rich medium, the yield of recombinant MNEI on the ac-CW-based medium seems higher than in the reference condition. Densitometric quantification of MNEI by western blot, in comparison with pure protein samples, allowed us to estimate a yield of 270 ng MNEI/10 μg total proteins 2 h after induction, i.e. almost twice the observed yield in G-M17 medium. The yield of protein per liter of culture medium was nonetheless comparable to that in G-M17, i.e. ~0.49 mg, because of the lower biomass reached in ac-CW-based growth conditions. Interestingly, when the recombinant strain carrying the pNZ8148-*MNEI-ec* vector was grown on the ac-CW-based medium, no protein production could be detected (Additional file 5: Figure S4).

Expression of MMP-9 on CW-based medium

As a proof of concept, to demonstrate the suitability of the ac-CW-based medium to produce recombinant proteins in *L. lactis*, we tested its efficiency on a previously characterized system, namely *L. lactis* NZ9000 *clpP*$^{-}$ *htrA*$^{-}$ carrying the pNZ8148-*MMP-9* vector coding for the catalytic domain (Phe107-Pro449, ~40 KDa) of metalloproteinase 9 (MMP-9) [44, 45]. Figure 4 shows the comparison between the growth curves in G-M17 and in ac-CW with 0.05% yeast extract. The comparison of the growth parameters for the two conditions is provided in Table 3. Compared to the reference condition in rich medium, we observe again the substantial reduction of the final biomass accumulation. Nonetheless, when MMP-9 expression was induced, with 10 ng/mL nisin

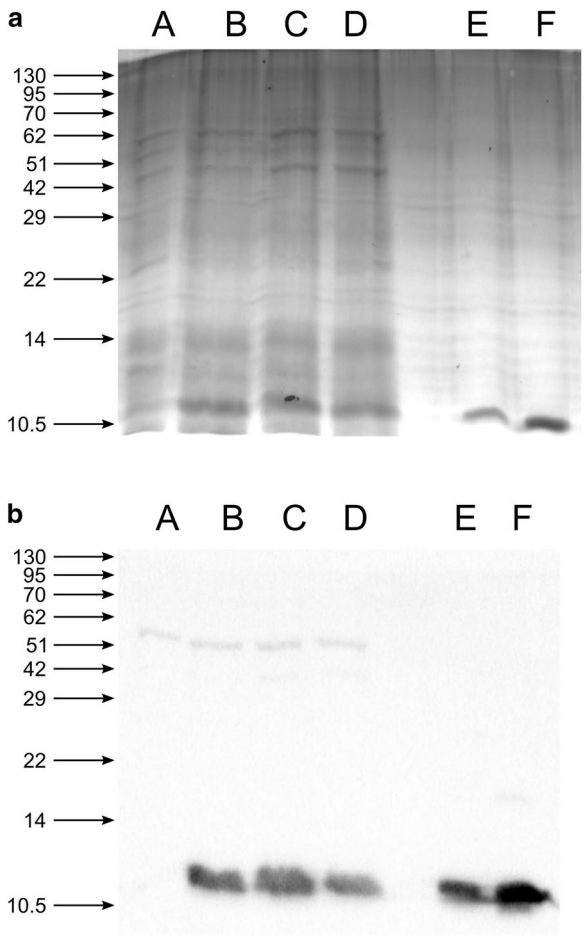

Fig. 3 Production of recombinant MNEI in the ac-CW based medium. Coomassie-stained SDS-PAGE (**a**) and western blot (**b**) of the total protein extract (10 µg) from *L. lactis* NZ9000 carrying the pNZ8148-*MNEI-II* and growing on ac-CW + 0.05% yeast extract. A: no induction; B: 2 h post-induction; C: 4 h post induction; D: 16 h post induction; E: MNEI standard, 200 ng; F: MNEI standard, 500 ng

after 2 h, comparable production of recombinant protein was obtained, as visible in Fig. 5, which shows the western blots of the total protein lysates obtained at different times post-induction in the two conditions examined. Maximal accumulation of the recombinant target seems only slightly delayed compared to the reference condition.

Discussion

The possibility of reintroducing waste materials in the productive cycle is a central issue of bioconversions and of the development of circular production strategies. The problem of handling the by-products of dairy industry has been long known: cheese whey (CW), the main by-product of cheese manufacturing, is a highly polluting waste, due to its high biological and chemical oxygen demand, which originates mostly from the residual lactose, but also from appreciable proteins and lipids content (Table 1) [29, 31]. Several strategies have been proposed to exploit these nutrients, reconverting them in added value compounds, often making use of LAB and of their ability of constitutively thrive on lactose. The growth of LAB on CW-based or CW-containing media, has allowed for the obtainment of various industrially relevant compounds, such as bacteriocins, ethanol, organic acids, often with the aid of metabolic engineering [18, 20, 21, 28, 30]. The utilization of CW as a suitable growth substrate, though, often requires preliminary treatments, which can range from complete deproteinization [20], to the supplementation of nutrients, with the addition of substantial amounts of peptone and yeast extract [21], to treatment with proteases or proteolytic microorganisms [32], and can greatly affect the sustainability and viability of the process. In this paper, we have described for the first time the possibility of using a CW-based medium also for the production of recombinant proteins in *L. lactis*. In the attempt of defining an industrially viable process, we have tried to keep the number of preliminary steps on CW prior to the cultures to a minimum, since pH adjustment and supplementation of catalytic amounts, i.e. 0.05%, of yeast extract have proven enough to produce acceptable biomass accumulation of *L. lactis* NZ9000. Using such a medium, it has been possible to also sustain recombinant protein production with the nisin-inducible NICE system. We have used the pNZ4184 vector carrying the gene coding for the sweet protein MNEI. This protein has been selected for its potential industrial application as a sugar replacer, which has motivated several efforts in the past to develop strategies for recombinant production [35]. Its production in *L. lactis*, a GRAS host, could facilitate the use of the protein as a food additive. Our results clearly indicate that the choice of the appropriate codon usage is a fundamental requisite for the correct expression of the protein, contradicting previous reports on the production of brazzein [46], for which *E. coli* optimized gene corresponded to higher production yields. In the case of MNEI, when the *E. coli* optimized gene was used, protein production was completely abated in the CW-based medium. This result could be related to the specific algorithm used for the *E. coli* codon usage optimization. The coding sequence was optimized to always use the same triplet for each codon, i.e. the most frequently occurring in the *E. coli* coding sequences. In almost 18% of the MNEI coding sequence, the *E. coli* preference is just the opposite of the *L. lactis* one, thus resulting in a coding sequence in which 18% of the codons are classified as "rare" for *Lactococcus*. Interestingly, *L. lactis* is one of the few microbial species for

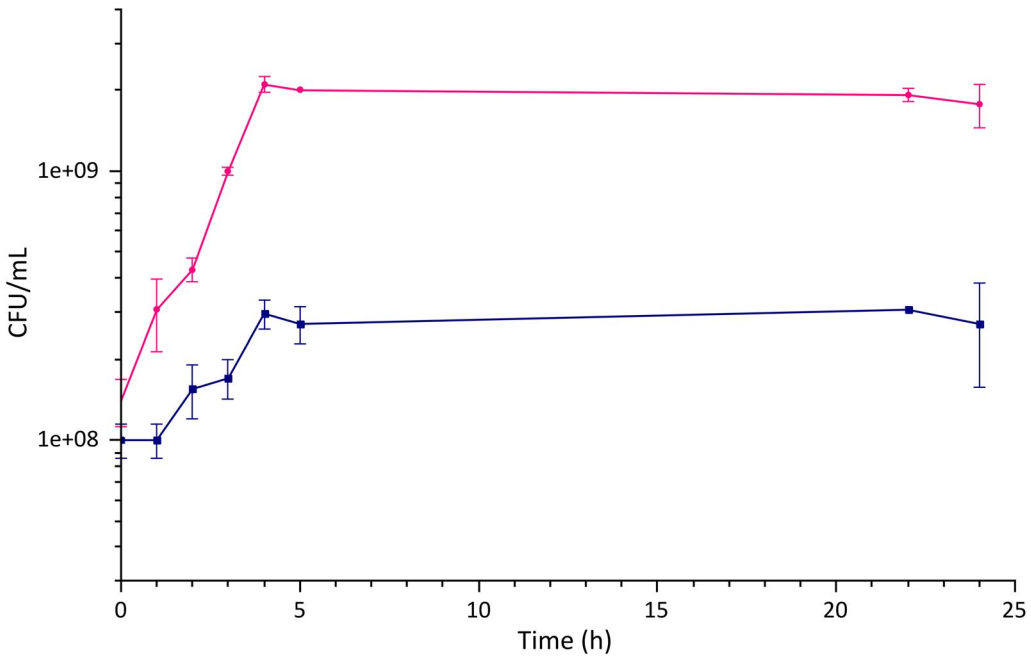

Fig. 4 Comparison of the growth of *L. lactis* NZ9000 *clpP*$^{-}$ *htrA*$^{-}$ in different media. The figure shows a comparison of the growth curve of *L. lactis* NZ9000 *clpP*$^{-}$ *htrA*$^{-}$ carrying the pNZ8148-*MMP-9* vector in the reference rich medium (pink curve), with the growth on ac-CW supplemented with 0.05% yeast extract (blue curve)

Table 3 Comparison of the growth parameters for *L. lactis* NZ9000 *clpP*$^{-}$ *htrA*$^{-}$ (pNZ8148-*MMP-9*) in G-M17 medium and ac-CW + 0.05% yeast extract

	Growth rate μmax (h^{-1})	Doubling time td (h)
G-M17	0.8 ± 0.1	0.8 ± 0.1
Ac-CW + 0.05% yeast extract	0.6 ± 0.2	1.2 ± 0.2

which a complete tRNAome determination was carried out, although in a growth condition investigated quite different from the two tested in the present work.

The general applicability of the CW-based medium has been demonstrated by producing the recombinant catalytic domain of MMP-9, using the previously described system *L. lactis* NZ9000 *clpP*$^{-}$ *htrA*$^{-}$ carrying the pNZ8148 vector coding for the catalytic domain of MMP-9 [44, 45]. Also in this case, recombinant protein production was comparable to the expression in standard rich medium. All the experiments described in this paper have been conducted in a laboratory set up, applying minimal control on the growth parameters, therefore with sub-optimal efficiency. The cultures on ac-CW-based medium always produced lower biomass than the corresponding control cultures in the standard medium. Even so, the yield of recombinant MNEI per liter of culture medium was comparable in the two conditions, due to the observation of higher titers of the target protein in the total protein extract. We can forecast that the introduction of controlled oxygenation and pH alone will compensate for the decrease in biomass accumulation. The production of organic acids throughout the fermentation, coupled to the lack of buffering capability of CW, causes in fact a quick drop in the culture pH to lethal values, which halts biomass accumulation. Moreover, ac-CW medium, as proposed in this paper, contains an unbalanced carbohydrates to proteins ratio that could be reduced by slight modification of the medium formulation. Optimization of these parameters and the use of fermenters will allow greater recoveries both in biomass and recombinant proteins.

Conclusions

We have presented a new strategy for the production of two recombinant proteins in two strains of *L. lactis* on a growth medium based solely on dairy by-products. Through minimal manipulation and the addition of minimal quantities of yeast extract, it has been possible to sustain microbial growth and to induce protein production levels comparable, if not superior, to the production in rich medium. The simplicity of the process makes it suitable for the application on the industrial scale and

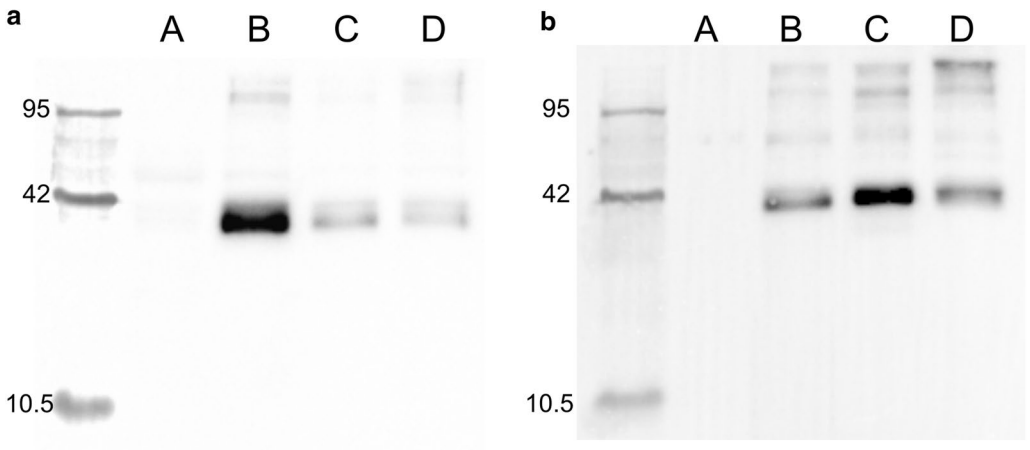

Fig. 5 Production of recombinant MMP9 in the G-M17 and ac-CW based medium. The figure shows a comparison of the western blots of 10 µg of total protein extract of cells of *L. lactis* NZ9000 *clpP*$^{-}$ *htrA*$^{-}$ transformed with the pNZ8148-*MMP9* vector after culture in rich medium (**a**) or in ac-CW supplemented with 0.05% Yeast extract (**b**). A: no induction; B: 2 h post-induction; C: 4 h post induction; D: 16 h post induction. While protein expression levels seem comparable in the two cases, maximum production appears slightly delayed in the case of the CW derived medium

prone to wide margins of improvement through the control of the growth parameters.

Methods

Bacterial strains and plasmids

Lactococcus lactis NZ9000 (*pepN::nisRnisK*) (NIZO) and the double mutant *L. lactis* subsp. *cremoris* NZ9000 *clpP*$^{-}$ *htrA*$^{-}$ [44, 45, 49] used in this study were maintained as frozen glycerol stocks at −80 °C. The Cm^{R} pNZ8148 plasmid (NIZO), under *nis*A promoter control, was used in this work. MNEI was expressed in *L. lactis* NZ9000, while the catalytic domain of metalloproteinase 9 (MMP-9), from *Bos taurus*, was produced, with a 6×His-tag, in *L. lactis* NZ9000 *clpP*$^{-}$ *htrA*$^{-}$ (*clpP-htrA*; erythromycin resistant (Em^{R})) (kindly provided by INRA, Jouy-en-Josas, France; patent n° EP1141337B1) [45, 49]. The gene sequences for MNEI with optimized codon usage for *L. lactis* (*MNEI-ll*) and *E. coli* (*MNEI-ec*) were purchased from Eurofins Genomics and received within commercial vectors. Both synthetic genes contained the *Not*I restriction enzyme site for the screening of the recombinant clones and were cloned into the pNZ8148 expression vector between the *Nco*I and *Hin*dIII restriction sites (Additional file 1: Figure S1). Plasmid isolation from *L. lactis* cells was achieved with the PureYield kit (Promega) after incubation of the cells with 5 mg/mL Lysozyme, 2 h, 37 °C.

Compositional analysis of CW

Cheese whey (CW) from the production of ricotta was obtained from "Caseificio Le Terre di Don Peppe Diana" and stored at −20 °C until used. Ac-CW was obtained by adjusting the pH of CW to 6.8, sterilization by autoclave and centrifugation (14,000×*g*, 30′, 4 °C) to remove the precipitate. Protein determination was performed by Bradford assay (Bio-rad). Carbohydrates determination was obtained by the Phenol/Sulfuric Acid assay [50]. Lipid determination was obtained by the Bligh & Dyer extraction, as reported [51].

Cell cultures

Lactococcus lactis was grown in M17 medium supplemented with 0.5% D-glucose (G-M17), in ac-CW and in ac-CW supplemented with 0.05% Yeast extract. Culture were kept static at 30 °C. Typically, 30 mL cultures were performed in 50 mL tubes. For recombinant strains, the growth medium was supplemented with 5 µg/mL chloramphenicol. In general, overnight cultures of *L. lactis* in G-M17 were diluted into 30 mL of culture medium to an OD600 of 0.1 Bacterial growth rates in G-M17 and in ricotta cheese whey were measured by plating appropriate dilutions of the culture suspension on G-M17 agar plates and counting the CFU after incubation at 30 °C for 24 h.

Production of MNEI and MMP-9 in *L. lactis*

L. lactis NZ9000 competent cells were transformed by electroporation with either the pNZ8148-*MNEI-ll* or pNZ8148-*MNEI-ec* vector [52]. Electroporation was performed with a Gene Pulser from Bio-rad fitted with 2500 V, 200 X and 25 µF in a pre-cooled 2 cm electroporation cuvette. Samples were then incubated for 2 h at 30 °C in 900 µL restorative medium (G-M17 with 20 mM Mg_2Cl_2 and 2 mM Ca_2Cl_2). The electroporation mix was centrifuged for 10 min at 10,000×*g* at 4 °C and the pellet

was resuspended in 100 µL of G-M17 media and plated. Recombinant cells were grown as described in the previous section. Expression of the MNEI gene was induced by administration of 10 ng/mL nisin in the mid-exponential phase, i.e. 2 h into the growth. At 2, 4 or 16 h post induction, 10 mL from the culture were harvested and the cells were pelleted by centrifugation (10,000×*g*, 4 °C, 30′), washed twice with cold PBS, resuspended in 1 mL of 50 mM sodium acetate buffer, pH 5.5, and disrupted by sonication. The MMP-9 recombinant production was carried out in *L. lactis* NZ9000 *clpP*$^{-}$ *htrA*$^{-}$ recombinant with pNZ8148-*MMP-9* [44], following the same conditions previously described for MNEI production.

SDS-PAGE and Western blot

Total protein content of the sonicated fractions was estimated by Bradford assay using BSA as standard. The purified recombinant MNEI from *E. coli* (MW ~ 11 kDa), obtained as described in [53], was used as positive control. Samples of 10 µg total protein extract were loaded on 12% SDS-PAGE and then blotted onto Immobilon-P transfer membrane (EMD, Millipore Corporation, USA). Membranes were incubated with an rabbit anti-Y65R-MNEI antibody [36] (1:200, Primmbiotech, courtesy of Dr. Nunzia Scotti) and subsequently with a HRP-conjugated anti-rabbit antibody (1:50,000, BioFX Laboratories). Detection of the His-tagged MMP-9 expression in *L. lactis clpP*$^{-}$ *htrA*$^{-}$ was also performed by western blot. The membranes were incubated with anti-poly-His Peroxidase conjugate (1:2000). In both cases the signal was revealed by enhanced chemiluminescence (ECL) kit (Biorad, Clarity™ western ECL Substrate) and recorded on a ChemiDoc™ MP Imaging System (Biorad).

Additional files

Additional file 1: Figure S1. Alignment of the *MNEI-ec* and *MNEI-ll* gene sequences. Restriction sites used for cloning are indicated in blue (NcoI and Hind III). A NotI site (red) was included for plasmid screening.

Additional file 2: Figure S2. Effect of the codon usage on recombinant protein production. Western blot (B) of the total protein extract (10 µg) from *L.lactis* NZ9000 carrying the pNZ8148-*MNEI-ec* vector. A: no induction; B: 2 h post-induction; C: 4 h post induction; D: 16 h post induction; E: MNEI, 50 ng; F: MNEI, 200 ng; G: MNEI, 500 ng.

Additional file 3: Figure S3. Evolution of the pH during cell culture. Plot of the pH vs time during the growth of *L. lactis* NZ9000 in G-M17 medium (blue curve) and in ac-CW + 0.05% yeast extract (red curve).

Additional file 4: Table S1. Composition of the G-M17 medium.

Additional file 5: Figure S4. Effect of the codon usage on recombinant protein production in CW-based medium. Western blot (B) of the total protein extract (10 µg) from *L.lactis* NZ9000 carrying the pNZ8148-*MNEI-ec* vector growth on ac-CW + 0.05% yeast extract. A: no induction; B: 2 h post-induction; C: 4 h post induction; D: 16 h post induction; E: MNEI, 50 ng; F: MNEI, 200 ng; G: MNEI, 500 ng.

Abbreviations

CW: cheese whey; ac-CW: autoclaved/centrifuged cheese whey; NICE: nisin controlled gene expression system.

Authors' contributions

MB carried out the experiments, performed the data analysis and wrote the manuscript; AC and AP performed experiments and data analysis, SL designed the experiments, performed the data analysis and wrote the manuscript; EGF, EP, AA and MMC contributed to experimental design, data analysis and revised the manuscript; DP and MLT supervised the study, participated in its design and data analysis and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated during this study are included in this article, and all material is available upon request.

Consent for publication

All authors approved publication.

Ethics approval and consent to participate

Not applicable.

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