Tailor made expression hosts depleted in protease activity for recombinant protein production

Project acronym: PRODuCE
Project no: EIB.12.037
Dr. Andreas Schiermeyer, Fraunhofer IME

Project partners

- **C. Mark Smales** *(Industrial Biotechnology/School of Biosciences/University of Kent/Canterbury/UK)*
- **Christoph Heinrich** *(Xell AG/Bielefeld/Germany)*
- **Rita Abranches** *(Plant Cell Biology/ITQB/Oeiras/Portugal)*
- **Renier van der Hoorn** *(Plant Chemetics/MPIPZ/Germany)*
- **Andreas Schiermeyer** *(Plant Biotechnology/Fraunhofer IME/Aachen/Germany)*

- **Total project budget: 1.69 M €**
Background

- Recombinant proteins represent a fast growing class of pharmaceuticals with annual sales of 140 billion USD (2013)
- Products comprise vaccines, blood factors, hormones, growth factors and monoclonal antibodies (mAb)
- Several biopharmaceuticals have blockbuster status with annual sales of >1 billion USD

Introduction

• **Project objectives**
  - Monitoring proteolytic activities in various production hosts for biopharmaceuticals
  - Comparing classical (CHO) and emerging (plants) production platforms
  - Identification of specific proteases involved in target protein degradation and approaches to minimize their activity

• **General project approach**
  - Identification of a target protein: anti-HIV mAb 2F5
  - Screening a small molecule library of protease inhibitors
  - Activity-based protein profiling to identify proteases
  - Co-expression of protease inhibitors/gene silencing
Technical overview

- Monitoring mAb 2F5 degradation in spiking experiments (spent culture media, cell extracts)
- Screening small molecule protease inhibitors (~80 subst.)
- Identification of proteases by ABPP and mass spectrometry
Small molecule inhibitor screening

Spiking experiment in tobacco BY-2 cell culture supernatant indicates involvement of serine proteases in the degradation process of the antibody heavy chain (HC).


Pepstatin: inhibitor of aspartic proteases
E-64: epoxide inhibitor of cysteine proteases
GM6001: hydroxamate inhibitor of MMPs
PMSF: sulfonyl fluoride inhibitor of serine proteases
DFP: fluorophosphonate inhibitor of serine proteases
Activity-based protein profiling (ABPP)

Identified proteases (plants):
- Aspartic proteases (A1 family; MEROPS database)
- Cysteine proteases (C1 family)
- Metalloproteases (M1, M16, M17)
- Serine proteases (S8, S9, S10, S28 family)

Identified proteases (CHO cells)
- Cysteine proteases (C1 family)
- Serine proteases (S1 family)
### ABPP in *N. benthamiana*

#### Table: Selected Accessions

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#### Figure: Western Blot

- **E64**: Untreated: - ++, Agroinfiltrated: ++ ++
- **DCG04**: Untreated: + +, Agroinfiltrated: + +

**SyproRuby**

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[www.era-ib.net](http://www.era-ib.net)
ABPP in CHO supernatant

Papain-like cysteine proteases (C1 family) are active in CHO spent culture medium under **acidic** conditions.
Detection of proteases by 2-DE (CHO)

- Host cell proteins (HCP) of industrial CHO cell culture supernatants were investigated
- HCP profile was broadly similar across the panel
- Actual amounts of some specific HCPs differed
- Also identified proteases exhibiting differences in abundance

Detection of proteases in culture medium by zymography (CHO)

- High activity of proteases in serum-containing medium and culture supernatant
- Much less protease activity in culture supernatants of chemically defined ERA-GM medium
- Implementation of special components (e.g. salts, chelators, …) in ERA media can further reduce protease activity

Active proteases are detected by zymography:
Proteins of culture supernatants are separated under by native electrophoresis in a PAA gelatin matrix.
Clear zones indicate gelatin degradation by proteases.
CHO adaptation (AM) and growth medium (GM) development

Initial experiments: no growth

Successful medium variants after several development cycles

Medium development:
- Challenging due to requirements for various applications:
  - Serum removal
  - Adaption to suspension
  - High performance production process

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Cell line generation from single cell CHO clones

- Example: generation of an Erythropoietin (EPO)-producing cell line based on adapted project host cell
- ERA-AM, ERA-GM and feed solution will be further evaluated aiming for commercialization of these media
Transient gene expression in *N. benthamiana*

**Applications**
- Protein production: e.g. ZMapp mAb cocktail against Ebola virus
- Rapid construct testing: RNAi; co-expression of protease inhibitors

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Rapid construct testing in *N. benthamiana*

- Approx. 50 proteases identified in the apoplast (some upon *A. tumefaciens* infiltration)
- 11 active, secreted Cys proteases identified by activity-based proteomics
- Cys protease inhibitor prevents cleavage of 2F5 antibody HC *in vivo*
- RNAi: 2/11 proteases crucial for POI cleavage; 5/11 crucial for plant survival
Stable expression of Kazal-like serine protease inhibitor in tobacco BY-2 cells

- Tobacco BY-2 cells have been stably transformed with Kazal-like serine protease EPI 10 from *P. infestans* to inhibit subtilases
- Clones that actively secrete EPI 10 inhibitor show less mAb 2F5 heavy chain degradation
- In parallel cell lines were generated that stably express serpin H1 from *M. sexta*
- Obstacle: clones with high expression levels display reduced growth performance
Summary

• Active proteases acting on biopharmaceuticals have been identified in mammalian (CHO) and plant production systems (*M. sativa, N. benthamiana, N. tabacum*)

• Strategies have been developed to suppress these proteolytic activities (RNAi, inhibitor expression, gene disruption, medium development)

• Development of improved production hosts is ongoing

• Follow-up projects
  - GreenProteases (ERC consolidator grant to Renier van der Hoorn)
  - ERA-IB INNOVATE (C. Mark Smales, co-ordinator)
Dissemination activities

• **Publications:**
  
  
  
  

• **Conference contributions**
  
  • Abranches R.: The model legume Medicago truncatula expression system: Towards high-yield production of recombinant proteins in cell suspension cultures. 1st ISPMF conference. June 2014, Berlin, Germany
  
  • Hogwood, C.E.M et al.: Mammalian CHO cell line protease activity and their impact upon secreted recombinant protein authenticity. BEBPA’s 3rd Annual Host Cell Protein Workshop (2015), San Francisco, USA
  
  • Santos, R.B. et al.: Host engineering of *Medicago truncatula* cell cultures for the improved production of recombinant proteins, RPP8, 22.-24.04.2015, Palma, ES
  
  • Mandal, M.K. *et al.*: Coping with proteolytic degradation of recombinant proteins produced in tobacco BY-2 cells. PBVAB, 08.-10.06.2015 Lausanne, CH
General Evaluation

• **Benefits of international collaboration**
  - Investigation of multiple production systems in parallel
  - Lab visits of researchers for training purposes
  - Open exchange of methods, materials and ideas
  - Training of PhD students in a collaborative international setting

• **Comments, feedback to ERA-IB**
  - Building a research consortium would be facilitated by the participation of more EU member states/funding agencies
Contact details

- C. Mark Smales: C.M.Smales@kent.ac.uk
- Christoph Heinrich: christoph.heinrich@xell.ag
- Renier van der Hoorn: renier.vanderhoorn@plants.ox.ac.uk
- Rita Abranches: rita@itqb.unl.pt
- Andreas Schiermeyer: andreas.schiermeyer@ime.fraunhofer.de
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