

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

ERA-IB-2 final conference, Berlin, 16./17.02.2016

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

Table 3 The 20 top-selling biopharmaceutical products in 2013

Ranking	Product	Sales ($ billions)[a]	Year first approved	Company
1	Humira (adalimumab; anti-TNF)	11.00	2002	AbbVie & Eisai
2	Enbrel (etanercept; anti-TNF)	8.76	1998	Amgen, Pfizer, Takeda Pharmaceuticals
3	Remicade (infliximab; anti-TNF)	8.37	1998	J&J, Merck & Mitsubishi Tanabe Pharma
4	Lantus (insulin glargine)	7.95	2000	Sanofi
5	Rituxan/MabThera (rituximab; anti CD20)	7.91	1997	Biogen-IDEC, Roche
6	Avastin (bevacizumab; anti-VEGF)	6.97	2004	Roche/Genentech
7	Herceptin (anti-HER2)	6.91	1998	Roche/Genentech
8	Neulasta (pegfilgrastim)	4.39	2002	Amgen
9	Lucentis (ranibizumab; anti-VEGF)	4.27	2006	Roche/Genentech, Novartis
10	Epogen/Procrit/Eprex/ESPO (epoetin alfa)	3.35	1989	Amgen, J&J, KHK

Walsh, G.: Biopharmaceutical benchmarks. *Nat. Biotechnol.* (2014)

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

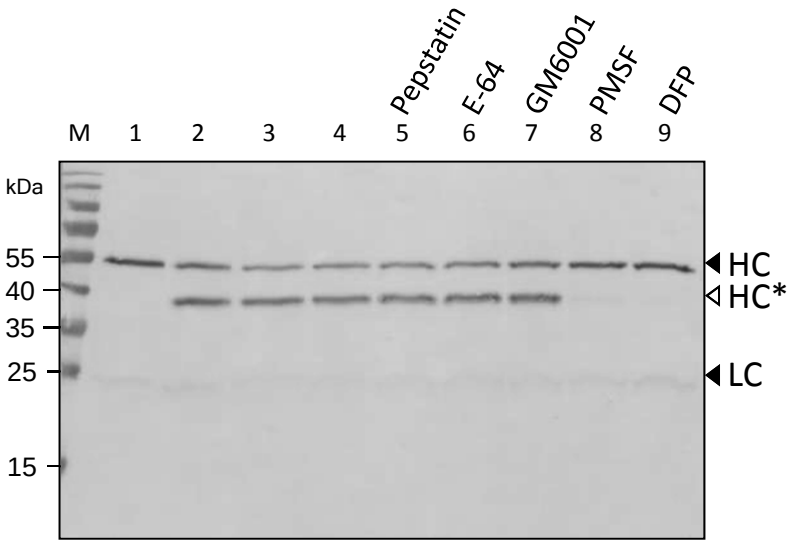
CHO cells

Plant suspension cells

Intact plants

- *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



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

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

Mandal, M., *et al*. (2014): Inhibition of protease activity by antisense RNA improves recombinant protein production in *Nicotiana tabacum* cv. Bright Yellow 2 (BY-2) suspension cells. Biotechnology Journal

Activity-based protein profiling (ABPP)

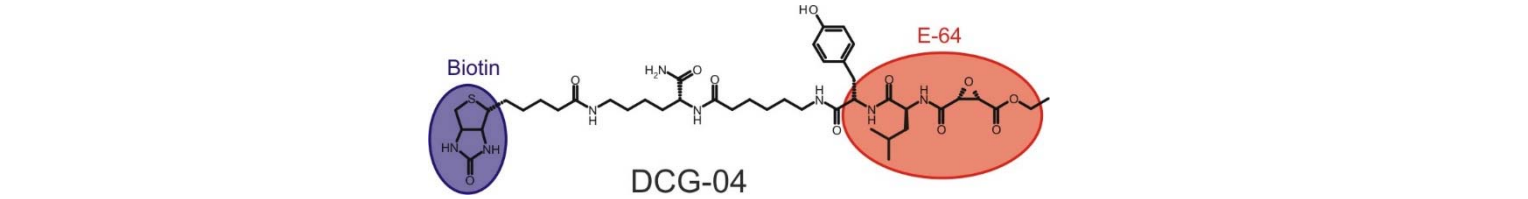
Identified proteases (plants):

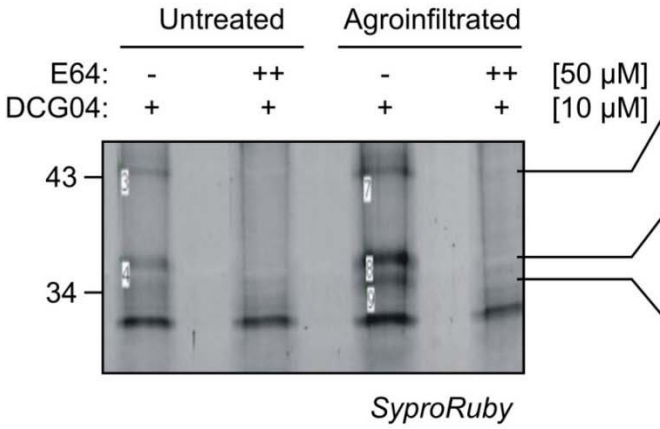
- Aspartic proteases (A1 family; MEROPS database)
- Cysteine proteases (C1 family)
- Metalloproteases (M1, M16, M17)
- Serine proteases (S8, S9, S10, S28 family)

Identified proteases (CHO cells)

- Cysteine proteases (C1 family)
- Serine proteases (S1 family)

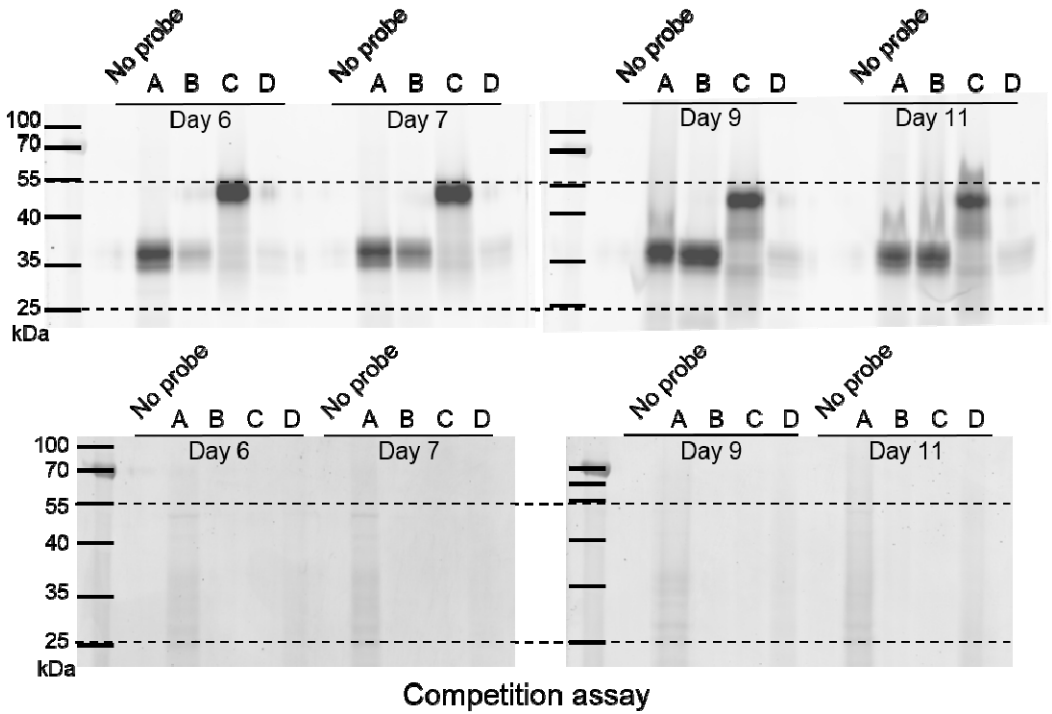
ABPP in *N. benthamiana*





Accession	Class	Untreated	Agroinfiltrated
NbC26208175g0003.1	RD21	49	31
NbS00040506g0007.1	RD21	2	12
NbC26208175g0003.1	RD21	10	7
NbS00040506g0007.1	RD21	2	2
NbS00008667g0012.1	CTB	9	28
NbS00003179g0007.1	RD21	0	5
NbS00035145g0007.1	CTB	0	2
NbS00003743g0028.1	XBCP	0	4
NbS00018127g0007.1	XCP	1	1
NbS00040834g0005.1	RD19		9
NbS00010435g0011.1	RD19		4
NbS00009882g0003.1	ALP		14
NbS00018589g0007.1	CTB		14

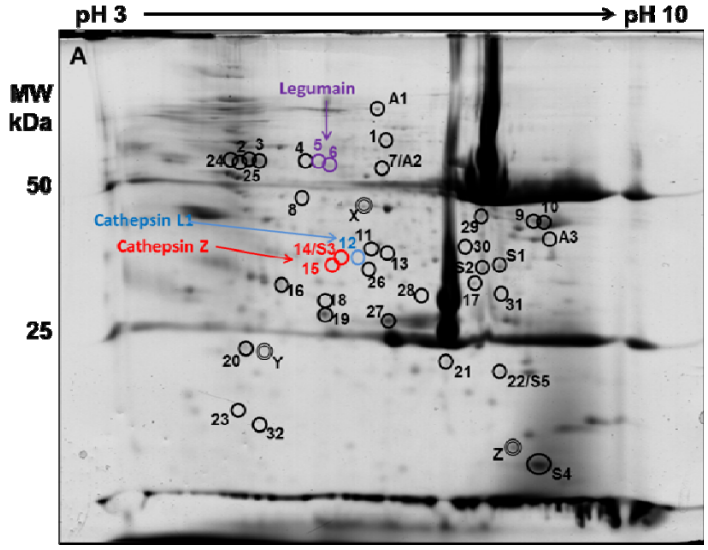
ABPP in CHO supernatant



A: PLCPs
B: Cathepsin B
C: Legumains
D: PLCPs

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

Detection of proteases by 2-DE (CHO)

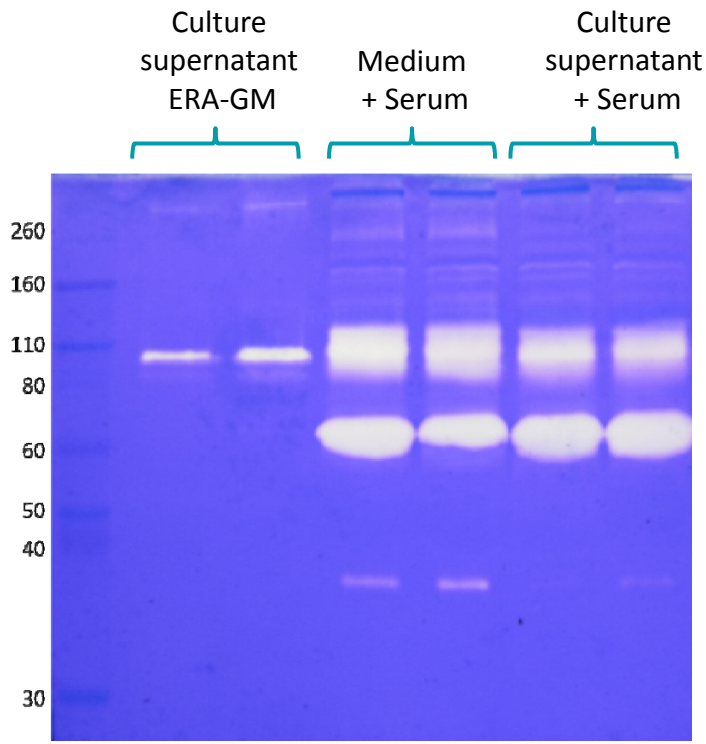


- Host cell proteins (HCP) of industrial CHO cell culture supernatants were investigated
- HCP profile was broadly similar across the panel
- Actual amounts of some specific HCPs differed
- Also identified proteases exhibiting differences in abundance

Hogwood, C.E.M., Ahmad, S.S., Tarrant, R.D., Bracewell, D.G. and Smales, C.M. *Biotechnol. J.* 2015. DOI.10.1002/biot.201500010.

Detection of proteases in culture medium by zymography (CHO)





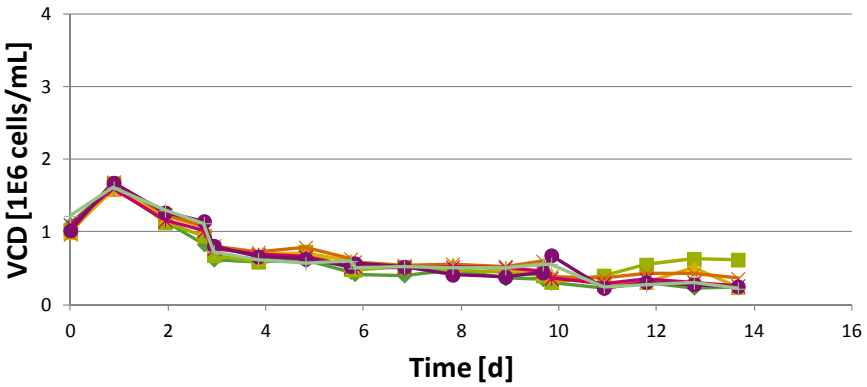
Active proteases are detected by zymography:
Proteins of culture supernatants are separated under by native electrophoresis in a PAA gelatin matrix.
Clear zones indicate gelatin degradation by proteases.

- 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 acitivity

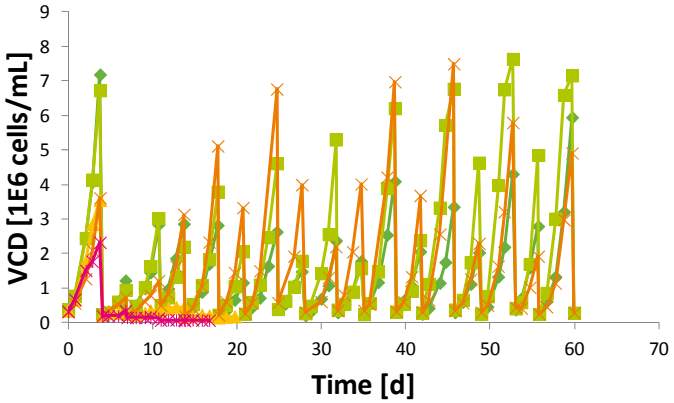
CHO adaptation (AM) and growth medium (GM) development



Initial experiments: no growth



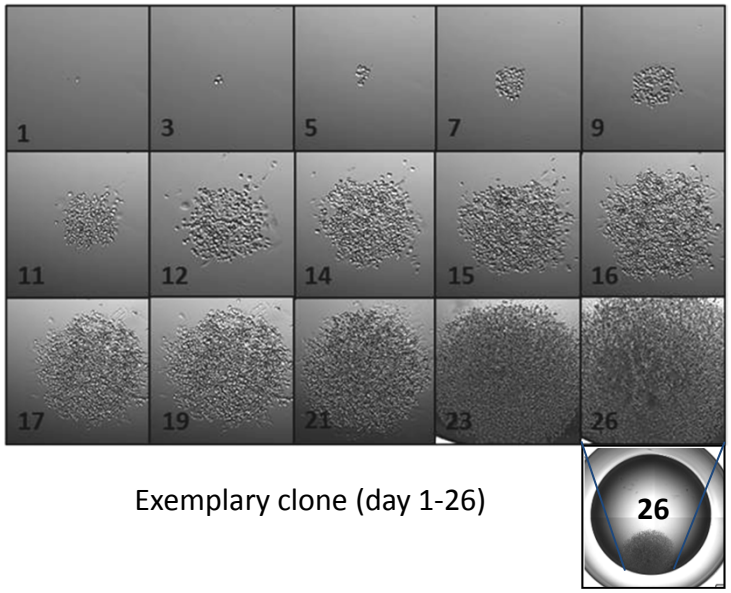
Successful medium variants
after several development cycles



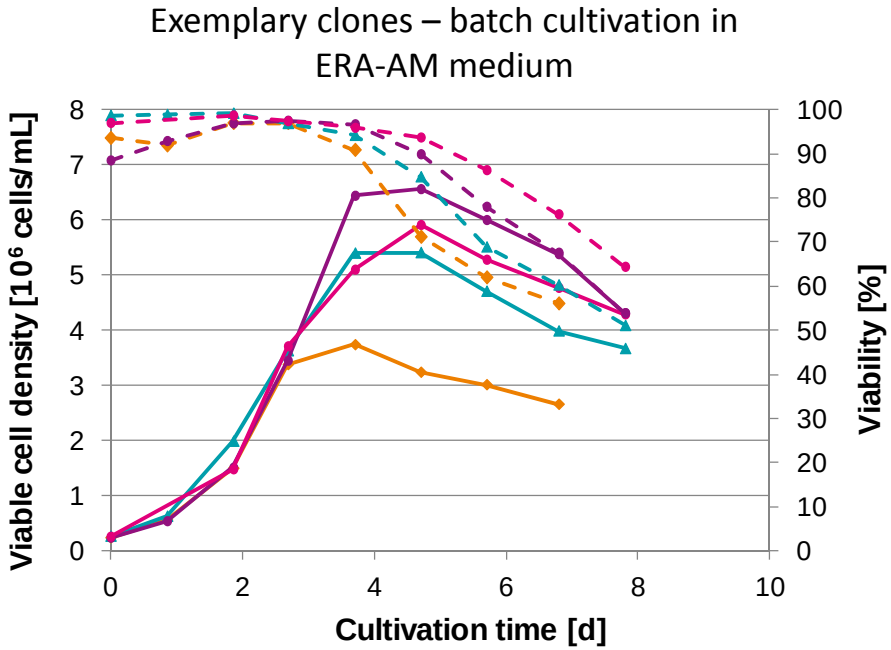
Medium development:

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

Cell line generation from single cell CHO clones

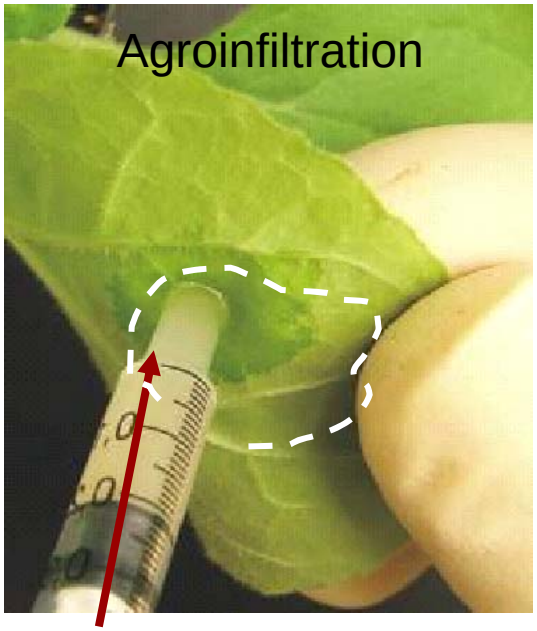


Exemplary clone (day 1-26)



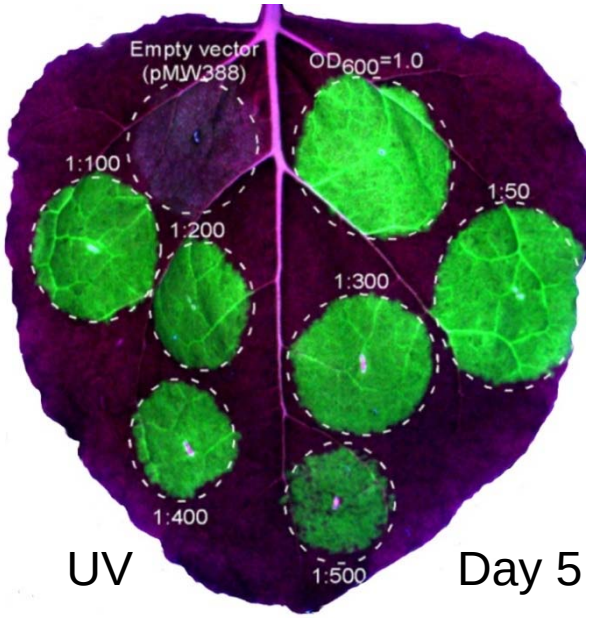
- Example: generation of an Erythropoietin(EPO)-producing cell line based on adapted project host cell
- ERA-AM, ERA-GM and feed solution will be further evaluated aiming for commercialization of these media

Transient gene expression in *N. benthamiana*



Agrobacterium tumefaciens carrying expression cassette

GFP expression

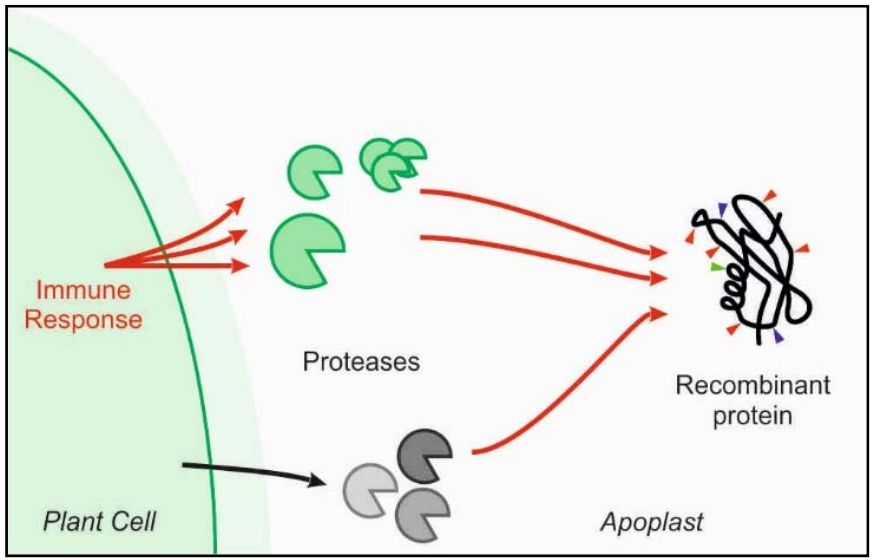


Applications

Protein production: e.g. ZMapp mAb cocktail against Ebola virus

Rapid construct testing: RNAi; co-expression of protease inhibitors

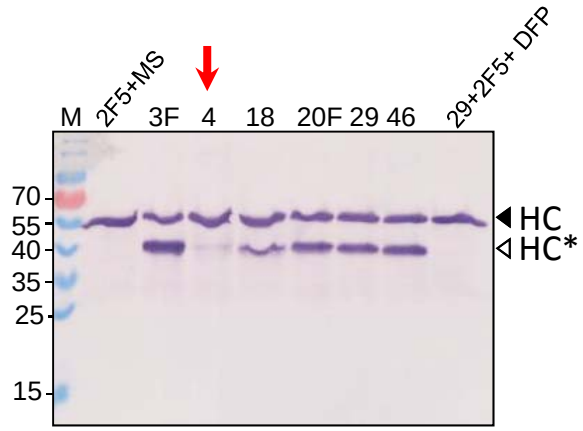
Rapid construct testing in *N. benthamiana*

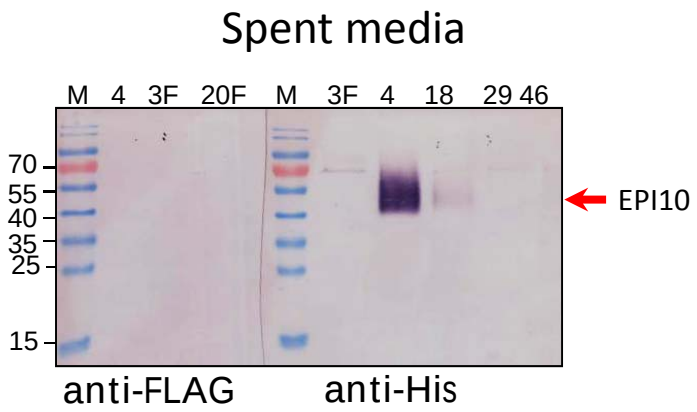


- Approx. 50 proteases identified in the apoplast (some upon *A. tumefaciens* infiltration)
- 11 active, secreted Cys proteases identified by activity-based proteomics
- Cys protease inhibitor prevents cleavage of 2F5 antibody HC *in vivo*
- RNAi: 2/11 proteases crucial for POI cleavage; 5/11 crucial 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:*
  - Mandal, M.K., *et al*. (2014): Inhibition of protease activity by antisense RNA improves recombinant protein production in *Nicotiana tabacum* cv. Bright Yellow 2 (BY-2) suspension cells. Biotechnology Journal
  - Hogwood, C.E.M., et al. (2015) An ultra scale-down approach identified host cell protein differences across a panel of mAb producing CHO cell line variants. *Biotechnol. J.* 2015. DOI 10.1002/biot.201500010
  - Mandal, M. K., et al. (2016): Tackling unwanted proteolysis in plant production hosts used for molecular farming. Frontiers in Plant Science. In press
  - Hogwood, C.E.M., et al. Protease profiling and activity assessment in Chinese hamster ovary cells. 2016. In preparation.
- *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: ritaa@itqb.unl.pt*
- *Andreas Schiermeyer: andreas.schiermeyer@ime.fraunhofer.de*

Acknowledgements



- Catherine E. M. Hogwood, University of Kent, UK
- Rita B. Santos, ITQB, PT
- Balakumaran Chandrasekar, MPIPZ, DE
- Manoj K. Mandal, Fraunhofer IME, DE
- Carolin Fritzenkötter, Xell AG, DE

University of Kent

MAX-PLANCK-INSTITUT FÜR PFLANZENZÜCHTUNGSFORSCHUNG

xell

ITQB INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA ANTÓNIO XAVIER/UNL Knowledge Creation

Fraunhofer IME