HETEROLOGOUS EXPRESSION OF NOVEL PROTEASES

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Abstract.

In view of the recent trend of developing environmentally friendly technologies the application of enzymes in until now environmentally harmful processes are coming more and more into focus. As biocatalysts, enzymes offer the large benefit that they work energy- and resource-efficient under comparatively mild conditions compared to the traditional chemical processes. Proteases belong to a large group of degradative enzymes that hydrolyze peptide bonds and account for about 60% of worldwide sales of industrial enzymes. They can be found in a variety of applications, e.g. as laundry detergent, as well as in leather, food and feed industry [1]. As constantly new application fields of these enzymes emerge, proteases with customized but robust characteristics are needed. In order to meet this demand, we underwent an approach to identify novel proteases by an in-silico search of genomes of putative protease-producing species, produce these candidate enzymes after optimization of the cultivation strategy in appropriate host organisms, *E. coli* and *P. pastoris* respectively, and determine their protein characteristics and enzymatic activities after purification. Some candidate proteases could not be produced in sufficient amount, most likely due to their intrinsic auto-catalytic activity resulting in fast degradation of the recombinant enzyme itself. Nevertheless, several proteases could finally be produced, which show high stability and activity over a broad pH and temperature range, making them promising enzymes for industrial applications.

Keywords: protease, recombinant protein production, Pichia pastoris

1 INTRODUCTION

Proteases form a large group of degradative enzymes and belong to the class of hydrolases, called peptide hydrolases. They catalyze the hydrolysis of peptide bonds in proteins by breaking down proteins or large peptides into fragments – smaller peptides up to dipeptides and single amino acids. Proteases are found ubiquitously, they occur in all organisms and viruses, cells and tissues. As intracellular enzymes they are involved in a variety of processes,

such as post-translational modification and activation of proteins as well as degradation of no-longer used or erroneous proteins. Extracellular proteases, which are secreted, are for the most part digestive enzymes, e.g. making protein sources in the close vicinity accessible for the organism [1].

Due to their diversity, there is an extensive field of industrial applications for proteases. They are mainly used as laundry detergents, in food and feed industry as well as in the medical field and in biotechnology. Commercially, microbial proteases are used most frequently, holding the largest market share [1].

In our attempt to find novel proteases with desirable features for industrial application we expressed predicted protease genes in the appropriate host organisms and determined their characteristics and enzymatic activities under different settings.

2 METHODS

2.1 GENERAL WORKFLOW

The major steps in identification and production of novel proteases are depicted in figure 1. Details will be described in the following sections.

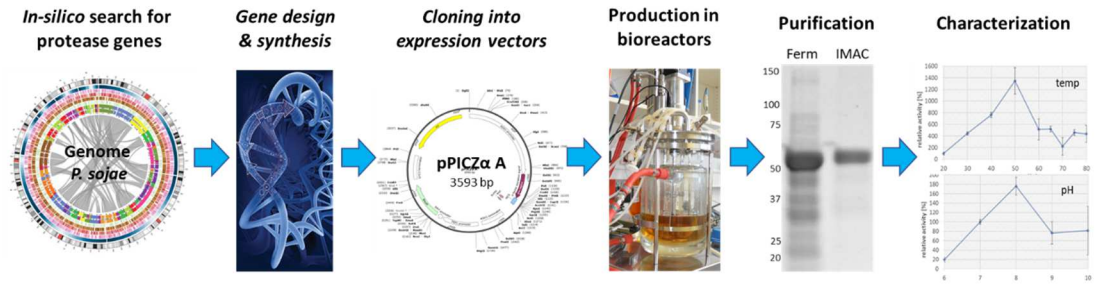

Figure 1. Workflow for the heterologous expression of novel proteases - from gene identification to characterization

2.2 IDENTIFICATION OF PUTATIVE PROTEASE SEQUENCES AND SYNTHESIS OF GENES

In order to identify novel proteases for industrial application, organisms were searched for, in which the occurrence of potent proteases could be highly assumed, like pathogens on protein-rich substrates. The genomes of these species were then analyzed for sequences with high similarity to known proteases. Candidate genes were subjected to codon optimization, specific for their subsequent production host, sequence tags were added to enable easier production and purification of the heterologous proteins and the such modified sequences were synthesized by an external company.

2.3 CLONING INTO EXPRESSION VECTORS AND EXPRESSION IN HOST ORGANISMS

Synthesized genes were first re-cloned into appropriate expression vectors. For bacterial genes the pET-vector system and *E. coli* as host organism were used, eukaryotic genes were cloned into pPICZα-vector and expression was done in *P. pastoris*. The integrity of the generated expression constructs was verified by sequencing. Transformation into *E. coli* and *P. pastoris* was done by heat-shock and electroporation, respectively.

Antibiotic selection markers were used to screen for positive transformants, which were then used for production runs. As in *P. pastoris* the expression cassette gets inserted into the genome, resulting in clones with different efficacy in protein expression, transformants were first screened by dotblot for expression of the protease (chemiluminescence detection of the His-tag) and only clones with strong signal were included into further analyses.

2.3.1 EXPRESSION OF PROKARYOTIC ENZYMES IN *E. COLI*

Bacterial proteases were expressed in the BL21(DE3) *E. coli* strain. Cultivation was done in LB-media containing ampicillin in shake flasks and expression was induced by addition of IPTG. After fermentation cells were harvested by centrifugation and subsequently disrupted by sonication to release the intracellular protein. As the expressed proteases formed inclusion bodies, solubilization, refolding and subsequent dialysis was required prior to a final purification step by immobilized metal-ion affinity chromatography (IMAC) using the His-tag of the recombinant proteins.

2.3.2 EXPRESSION OF EUKARYOTIC ENZYMES IN *P. PASTORIS*

For the production of eukaryotic proteases, the *P. pastoris* strain KM71H was used. Cultivation was done in shake flasks as well as controlled 2-l bioreactors. Media for the growth and the production phase were optimized to yield maximal biomass, inducibility with methanol and protein yield by using different carbon-sources and additives. Fermentation was performed in batch as well as fed batch mode. Proteases secreted into the media were first concentrated by cross-flow filtration and thereafter purified by affinity chromatography using the His-tag (IMAC).

2.4 PROTEIN CHARACTERISATION

Purified proteases were first subjected to an analysis by SDS-PAGE and Western blot to verify their molecular weight and monitor the purification progress. Their specific enzymatic activity was determined on several substrates. Promising enzymes were further analyzed concerning their activity and stability at different pH and temperatures to evaluate their applicability in the different fields.

3 EVALUATION

3.1 PROKARYOTIC PROTEASES

Two serine proteases from the soy bean pathogen Xanthomonas axonopodis were successfully produced in *E. coli* using the IPTG-inducible, T7-RNA polymerase based pET 28b+ system. Both proteases were expressed as inclusion bodies, which were solubilized and refolded prior to a final purification by IMAC (see Figure. 2). The proteases, with a molecular weight of 63 and 45 kDa, respectively, showed an activity on casein of up to 23 U/mg.

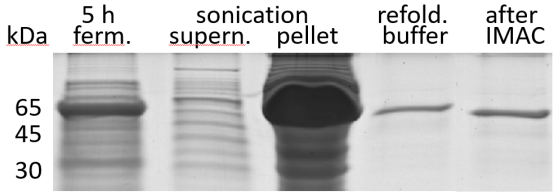

Figure 2. SDS-PAGE analysis of a 65-kDa serine protease from X. axonopodis. Protein samples after 5-hour expression in E. coli and the various purification steps

3.2 EUKARYOTIC PROTEASES

Protease genes originating from eukaryotic species were expressed in *P. pastoris*, using the methanol-inducible AOX-promotor of the pPICZα expression vector. Optimization of the fermentation process resulted in a synthetic media with reduced amount of glycerol, leading to a short batch phase with strong biomass production and low residual glycerol concentration at the end of the phase, and a continuous feed with sorbitol (as non-repriming carbon source) during the fed batch phase, which started simultaneous to the induction with methanol. Furthermore, ammonium as N-source and applied as pH-regulans was beneficial, casaminoacids and protease-inhibitor complex were added to fermentations with presumably autocatalytic enzymes, based on previous SDS-PAGE analyses.

Some of the purified proteases, e.g. a trypsin-like protease of *Phytophtora sojae*, an oomycete plant pathogen, or a chymotrypsin of *Periplanata americana*, the American cockroach, showed activity of up to 90 U/mg but also low stability. These enzymes might be potential candidates, but need further investigation to counteract their mechanism of (self-)degradation.

A chymotrypsin of *Solenopsis invicta*, the fire ant, a very aggressive predatory ant, was by far the most promising enzyme, showing an activity of > 5000 U/mg on casein. This enzyme was already described by Withworth et al. [2], who isolated endopeptidases from solenopsis in 1998, and Botos et al. [3], who published its structure in 2000, but so far, no heterologous expression and in-depth characterization of this highly active protease has been done. We looked at the activity and stability of this chymotrypsin at different temperatures and pH and

found it to be highly active in alkaline conditions with a maximum at pH 8, and increased activity at temperatures of up to 80°C with a maximum at 50°C (see Figure 3) and stability over a wide pH range from 6 to up to 10 (data not shown).

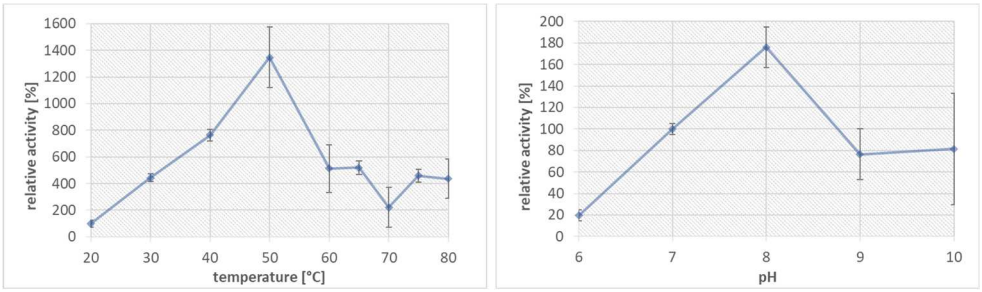

Figure 3. Enzymatic activity of a chymotrypsin of Solenopsis invicta at different temperatures (left) and pH (right), determined by FRET-Casein assay (relative to activity at pH 7 and 20°C).

Taken together, these characteristics make this chymotrypsin an interesting protease for a variety of applications at moderate temperatures and alkaline conditions.

4 CONCLUSION

In an attempt to identify novel proteases for industrial application we identified several candidate genes in the genomes of organisms, in which the occurrence of potent proteases could be highly assumed. A few of these genes have already been extracted from their original organisms in the past, but to our knowledge none has been recombinantly produced so-far. We were able to express most of these genes in *E. coli* or *P. pastoris* by optimizing the processes in batch as well as fed-batch fermentation. Some of the proteases showed good enzymatic activity but low stability and high presumably autocatalytic activity, necessitating further investigation to counteract this degradation. Of the remaining proteases, especially a chymotrypsin originating from the fire ant showed great potential as industrial enzyme due to its high enzymatic activity and stability at neutral to basic pH.

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