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Expression of a Codon-Optimized *Carica papaya* Papain Sequence in the Methylotrophic Yeast *Pichia pastoris*

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Abstract

The cysteine endoprotease papain is one of the most widely used plant proteases for industrial applications. However the traditional isolation of papain from the latex of papaya plants cannot cover the world-wide? demand. To increase papain production for industrial applications, several expression systems were studied in the last years for its recombinant production. While expression in *Eschericha coli* resulted in accumulation of insoluble protein, expression in baculovirus/ insect system and *Saccharomyes cerevisiae* resulted in low yields of soluble protein inadequate for large-scale production. Here we describe the heterologous expression of a synthetic codon-optimized propapain sequence in the *Pichia pastoris* strains X33 (Mut$^+$) and KM71H (Mut^s). The recombinant propapain could be expressed as soluble protein and secreted in the culture medium through the α-factor signal peptide. Highest activities were obtained in the Mut^s strain when cultivated in complex medium. After purification by Ni-NTA chromatography 463 mg/L recombinant propapain was obtained comparable to the so far highest reported propapain yields in *E. coli* after protein solublilization and refolding and with a specific activity similar to a commercial papain from papaya latex.

Keywords: Cysteine protease; Recombinant papain; Heterologous expression; Methylotrophic yeast; X33; KM71H

Introduction

The cysteine endoprotease papain (EC 3.422.2) is produced in the plant cell as a 345 amino acid long inactive preproprotein consisting of an 18 amino acid long signal-peptide and a 115 amino acid long propeptide. The catalytic active mature protein shows a broad specificity for peptide bonds and even hydrolyzes alkyl esters and amides.

To date the main source for papain is the latex of the papaya tree *Carica papaya*. From 45 g of plant material 12 g of enzyme can be extracted [1-3]. However papain is a minor component of cysteine proteases in the latex (5-8%) and to obtain highly pure preparations an excessive purification process is necessary [4]. In addition the isolated papain is unstable and has to be distributed quickly under cooled conditions without direct air contact [5]. Another disadvantage is the dependency of the papain production on the climate, growth and health of the papaya plants, which can lead to shortages in enzyme supply [5].

As an alternative several heterologous expression systems were investigated in the last years for the production of pure and high concentrated papain. Expression of the propapain sequence in *Escherichia coli* resulted in the formation of inclusion bodies [6]. Taylor et al. [7] were able to renature the insoluble protein to yields of ~ 3 mg/L. Choudbury et al. [8] obtained 400 mg/L of a His-tagged fusion propapain from *E. coli* by establishing an optimized purification and refolding protocol. However several undesired reactions are possible under such folding conditions, including autoproteolysis, active site oxidation and incorrect disulfide bond formation. The expression of soluble papain is *also/ furthermore* described in eukaryotic expression systems. Papain expression in the baculovirus/insect system resulted in the production of 0.3 mg soluble protein [9]. Higher concentrations were possible in the yeast *Saccharomyces cerevisiae* with up to 1.7 mg/L [10,11]. Recently Brömme et al. [12] described the *Pichia pastoris* system to be the most efficient host for the expression of human Cathepsin (also belonging to the papain like cysteine protease family). Despite several advantages of this expression system like the strong AOX1 promoter or the fermentation with high cell densities only Dufour et al. [13] and Versari et al. [14] used *P. pastoris* so far as an expression system for an engineered papain nitrile hydratase. Thereby Dufour et al. [13] were able to synthesize 3-5 mg/L of the papain nitrile hydratase proenzyme in *P. pastoris* [13]. However a synthetic codon-optimized sequence for the expression of recombinant papain in *P. pastoris* was not investigated so far.

In order to increase the expression of recombinant papain in methylotrophic yeasts we created a synthetic sequence of the papain precursor (propapain) gene with optimized codon-usage for *P. pastoris*. The recombinant propapain could be expressed as a His-tag fusion protein in the wild type *P. pastoris* strain (Mut+) and the methanol-utilization slow mutant (Mut^s) KM71H. After purification 463 mg/L enzyme was obtained showing a 1.4 times higher enzymatic activity towards the chromogenic peptide Z-phenylalanine-arginine-para-nitroanilide (Z-FR-pNa) than a commercial papain protease.

Methods

Strains and general cultivation conditions

The *Pichia pastoris* strains X33 (wild type; Mut$^+$) and KM71H (*arg4 aox1*::*ARG4*; Mut^S) (Life Technologies-Invitrogen, Carlsbad, California, USA) were used as expression hosts for recombinant protein production. For maintenance of the yeast cultures the strains were cultivated in YPD medium (yeast peptone dextrose medium: 10% yeast extract, 20% peptone and 20% glucose) at 28°C.

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Construction of the propapain expression plasmid

The propapain cDNA sequence described by Cohen et al. [15] (accession No M15230) was optimized for codon-usage in *P. pastoris*, splicing sites, GC-content, RNA secondary structure and content of restriction enzyme recognition sequences using the GeneOptimizer® expert software from Life Technologies-Invitrogen (Carlsbad, California, USA). Additionally a C-terminal polyhistidine (6xHis) tag was added. The optimized sequence was synthesized by GENEART AG (Regensburg, Germany). An EcoRI-NotI fragment containing the codon-optimized propapain sequence and the polyhistidine tag encoding sequence was excised from the backbone of the GENEART AG vector and cloned into the corresponding sites of the *P. pastoris* expression vector pPicZαA in frame with the α-factor signal peptide sequence and under control of the methanol inducible *aox1* promoter (Life Technologies-Invitrogen, Carlsbad, California, USA).

Transformation of *P. pastoris*

The constructed expression vector was linearized with SacI and transformed in the *P. pastoris* strains X33 and KM71H by electroporation. Transformants were selected on YPDS plates (YPD medium with 1 M sorbitol) with Zeocin concentrations of 100, 250 and 500 µg/mL and incubated for 2 to 3 days at 28°C.

Genotypic and phenotypic characterization of *P. pastoris* clones

The integration of the expression cassette into the genome was verified by colony PCR using the AOX3' and AOX5' primer pair according the EasySelect Pichia Expression kit manual (Life Technologies-Invitrogen, Carlsbad, California, USA). The methanol utilizing (Mut) phenotype of the clones was determined by comparing their growth on MD (minimal dextrose) and MM (minimal methanol) agar plates as described in the EasySelect Pichia Expression kit manual.

Expression of propapain in *P. pastoris*

Expression analysis experiments were performed in 250 mL baffled shaking flasks with 50 mL BMMY medium (buffered complex methanol medium: 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer (pH 6.0), 1.34% YNB (yeast nitrogen base), 4 X 10^{-5} % biotin, and 0.5% methanol), or 50 mL FM22 medium [16]. Both media were inoculated with a pre-culture grown overnight in BMGY medium (buffered complex glycerol medium: 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer (pH 6.0), 1.34% YNB (yeast nitrogen base), 4 X 10^{-5}% biotin, and 1% glycerol) to a starting OD_{600nm} of 1. The expression in BMMY medium was started directly after inoculation due to the presence of 0.5% methanol in the medium. For expression analysis in the FM22 medium, induction of papain expression was started after glycerol depletion with the addition of 0.5% methanol. The cultures were cultivated 2 to 3 days at 28°C and 200 rpm on a rotatory shaker. Addition of 0.5% methanol was repeated every 24 h. Every day, samples for OD measurement, SDS-PAGE and activity assay were collected.

Glycerol concentration was determined with the Glycerol assay kit from R-Biopharm AG (Darmstadt, Germany).

Propapain purification and western blot

Extracellular propapain was purified using Ni-NTA affinity chromatography. Therefore, 50 mL culture medium was first centrifuged for 10 min at 3500 g. 45 mL of the clear supernatant was supplemented with 5 mL binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) and 500 µL Ni-NTA agarose beads (Qiagen, Hilden, Germany). The mixture was incubated for 2 h on a rotatory shaker at 4°C and finally loaded on a chromatography column (20 mL bed volume). The Ni-NTA beads were washed with 3 column volumes of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). The propapain was eluted three times from the column with 250 µl elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Purified protein was separated by SDS-PAGE (12% acrylamide), blotted and immonodetected with anti-his primary antibody (1/2000 dilution). Protein concentrations were determined with the Bradford assay kit (Bio-Rad, Hercules, CA, USA).

In vitro activation and Z-FR-pNa hydrolysis assay

Propapain was activated *in vitro* with an activation buffer after Rozman et al. [17] containing 5 mM cysteine-HCl as reducing agent. For activation 48 µL cell-free culture medium or purified propapain was incubated with 2 µL activation buffer for 30 min at room temperature and stored on ice prior activity measurement. Enzymatic activity of the recombinant papain was measured in an end point assay with the chromogenic peptide Z-phenylalanine-arginine-para-nitroanilide (Z-FR-pNa; Enzo Life Science, Farmingdale, NY, USA) as substrate. Therefore 50 µL enzyme solution was incubated with 177 µL sodium phosphate buffer (0.1 M, pH 7.0) and 13 µL Z-FR-pNa (5 mg/mL) at 37°C and 600 rpm. The reaction was stopped after 2 min with 50 µL 50% acetic acid. The reaction mixture was centrifuged for 5 min at 13.000 rpm and the hydrolytic release of para-nitroanilide (pNA) was measured at 37°C with an extinction of 405 nm. Enzyme solution treated prior Z-FR-pNa addition with 50 µL 50% acetic acid was used as reference. For calibration pNA solutions of 0.8 to 12 mM were used. One Unit (U) was defined as the amount of enzyme catalyzing the hydrolysis of 1 µmol Z-FR-pNA per minute. All measurements were performed with two biological and two technical controls.

Results

Codon-optimization of the propapain sequence

To improve the expression of recombinant papain in *P. pastoris* the papain cDNA sequence described by Cohen et al. [15] was used as template for codon-optimization. Figure 1 shows an alignment of the original cDNA sequence and the codon-optimized sequence encoding the propapain (the codon-optimized DNA sequence is provided in the supplements in FASTA format). In addition, a polyhistidine encoding sequence was attached to the 3' end with two stop codons for translation termination.

Selection of recombinant *P. pastoris* strains for propapain expression

Several colonies were obtained after transformation of the propapain expression plasmid in the *P. pastoris* strains X33 and KM71H. Only clones selected on YPDS plates containing 500 µg/mL Zeocin were chosen for further investigation. Thereof, 10 clones from each strain were tested for genomic integration of the expression cassette by colony PCR. Further the methanol utilizing (Mut) phenotype of the clones was determined comparing their growth on glucose or methanol containing minimal medium. The KM71H strain used in this study shows a mutation in the *aox1* locus resulting in slow growth on methanol (Mut^s phenotype). Integration of the expression cassette in the wild type strain X33 results mostly in a methanol utilizing plus phenotype (Mut^+) showing normal growth on methanol. In rare events the integration can lead to a Mut^s phenotype by gene replacement at

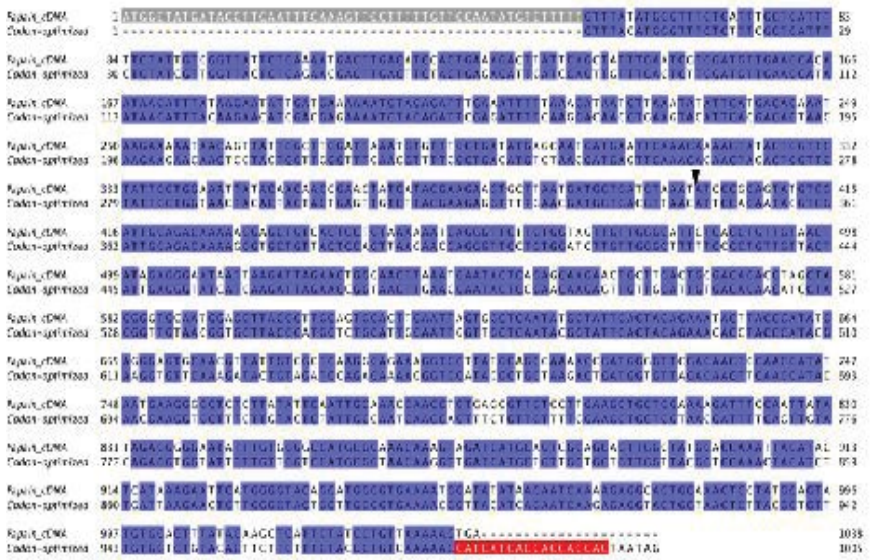

Figure 1: Nucleotide alignment of the full papain cDNA sequence described by Cohen et al.1986 (M15203) and the codon-optimized propapain sequence [15]. The sequence encoding the native signal peptide is highlighted in grey. The end of the propeptide encoding sequence is marked with a black triangle. The 3'end of the codon-optimized sequence is fused in frame with a polyhistidine (6x His) encoding sequence shown in red. The alignment was calculated with ClustalX and visualized with Jalview. Dark blue indicates conserved nucleotides between the two sequences.

the *aox1* locus [18]. However, all analyzed X33 clones in this study showed a Mut$^+$ phenotype (data not shown).

Heterologous expression of the codon-optimized propapain sequence

In a time course experiment papain production was investigated in both *P. pastoris* strains. Therefore two randomly chosen X33 (Mut$^+$) and KM71H (Mut^s) clones were cultivated in shaking flaks with complex (BMMY) or mineral salt (FM22) medium containing 0.5 % methanol for gene induction. Secretion of recombinant propapain during the cultivation was analyzed by measuring the proteolytic activity of cell-free samples of the culture medium after activation of the protease with cysteine.

Highest protease activities were measured during cultivation in BMMY medium with the Mut^s clones (Figure 2). The expression increased steadily over the three cultivation days, whereas expression in the Mut$^+$ clones stagnated (Figure 2A and 2B). Compared to the Mut$^+$ clones a 2.7 times higher specific activity (U/L*OD_{600}) was detected after 72 h (Figure 2B). No activity of secreted cysteine proteases was detected in cultivations with the untransformed X33 and KM71H strains (data not shown).

To simulate fermentation conditions in shaking flasks the same clones were cultivated in FM22 medium (Figure 2C and 2D). Similar to the fermentation process the cells were first cultivated as glycerol batch to increase the cell density. Then after glycerol depletion propapain expression was started with the addition of 0.5 % methanol. During the cultivation in FM22 medium an optical density (OD_{600nm}) of 55 could be reached (Supplementary Figure 1). Despite higher cell densities less recombinant papain was produced than in the complex medium BMMY (Figure 2B and D; Supplementary Figure 1). Interestingly, the Mut$^+$ and Mut^s clones showed similar expression capacities during FM22 cultivation.

Purification, activation and comparison with native papain

The secreted propapain was purified by Ni-NTA chromatography from a KM71H cultivation in BMMY medium. From 45 mL cell-free cultivation broth 463 mg/L enzyme could be obtained.

In situ papain is expressed as proteolytic inactive proenzyme, which has to be processed into its mature form by removal of its autoinhibitory propeptide region [19]. Propeptide removal and the subsequent activity of the enzyme can be performed at low pH by addition of acetate buffer at pH 4, or under reducing conditions by addition of dithiothreitol (DTT) or cysteine-HCl [8,12]. Choudhury et al. [8] compared the activation of recombinant propapain expressed in *E. coli* in the presence of DTT and cysteine. They observed best results after incubation with 20 mM cysteine for 30 min at 50°C. We could show that incubation with 5 mM cysteine for 30 min at room temperature was sufficient for papain maturation, which was determined by immunoblotting. In Figure 3 the mature papain appeared after activation as a single band with an expected size of 25 kDa indicating the successful removal of the 115 amino acid autoinhibitory propeptide. In addition, activation enhanced the proteolytic activity dramatically from 1.6 to 20.7 U/mg (Table 1).

The proteolytic activity of the recombinant protein was compared to a commercial papain enzyme from papaya latex (Sigma Aldrich, St. Louis, MO, USA). As shown in Table 1 the recombinant papain showed a 1.4 times higher specific activity (U/mg) towards the chromogenic peptide Z-FR-pNa than the native papain.

Discussion

In the presented work we describe the successful and high yield expression of a synthetic codon-optimized propapain sequence in the methylotrophic yeast *P. pastoris*. To our knowledge a codon-optimized papain sequence was never tested in methylotrophic yeasts, although a synthetic propapain sequence codon-optimized for yeasts was expressed by Vernet et al. [10] in *S. cerevisiae* under control of the

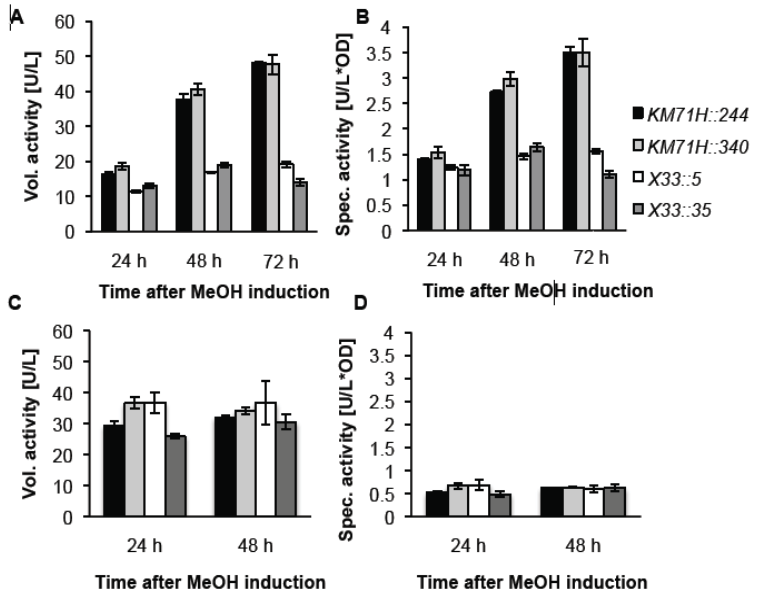

Figure 2: Papain activity in the supernatant of recombinant *P. pastoris* strains. Two randomly chosen strains from KM71H (*KM71H::244* and *KM71H::340*) and X33 (*X33::5* and *X33::35*) were investigated. Volumetric [U/L] and specific activities [U/L*OD] were determined in complex medium (BMMY) ((A) and (B)) 24, 48 and 72 hours and in mineral salt medium (FM22) ((C) and (D)) 24 and 48 hours after induction with 0.5% (v/v) methanol. The activity was determined after *in vitro* activation of the recombinant papain with Z-phenylalanine-arginine-para-nitroanilide (Z-FR-pNa) as substrate. Three biological and two technical replicates were used. OD=optical density at 600 nm.

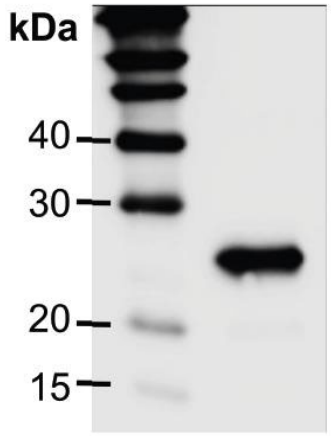

Figure 3: Western blot of the mature recombinant papain detected with anti-His antibodies. Propapain was heterologously expressed in *P. pastoris* as an α-factor-fusion protein. After secretion, the recombinant enzyme was purified from the cell-free culture medium by Ni-NTA chromatography and activated with cysteine-HCl. The mature protein shows a size of 25 kDa. Calculated size of the unprocessed propeptide is ~ 38 kDa.

Specific activity	Recombinant papain	Papain Sigma
Without activation	1.6 U/mg	n.d.
With activation	20.7 U/mg	14.9 U/mg

n.d. - not determined

Table 1: Specific activities of the recombinant papain expressed in *P. pastoris* and a commercial papain extracted from papaya latex.

constitutive active α-factor promoter and secreted through the α-factor signal peptide. However, the recombinant propapain retained within the *S. cerevisiae* cells. The authors suggested that the papain propeptide was responsible for the lack of secretion and the accumulation of the propapain in the yeast's vacuoles, since glycosylated, processed and active papain was found in the cell lysate. Secretion problems were also described for the *Zea mays* cysteine protease Mir1 heterologously expressed in *P. pastoris* [20]. The recombinant cysteine protease was fused to the PHO1 signal peptide for secretion, but the protease was found trapped in the cell membrane. Nevertheless, several other cysteine proteases were successfully expressed and secreted in *P. pastoris* directed through the α-factor signal peptide [21-23]. Also our results suggest that the α-factor signal peptide efficiently secreted the recombinant propapain into the culture medium thereby facilitating protein purification.

Choudhury et al. [8] studied the effect of reducing agents, incubation time and temperature on the processing and activation of recombinant papain expressed in *E. coli*. Best results were obtained after 30 min incubation with 20 mM cysteine at 50°C. At 37°C complete activation occurred after 2 h, whereas no activation was observed at 20°C even after prolonged incubation. In our experiments incubation for 30 min at room temperature with only 5 mM cysteine was already sufficient for complete papain processing (Figure 3).

In addition we studied the expression in two common Kursiv expression media, the complex medium BMMY and the mineral salt medium FM22 often used for fermentation (Figure 2). Cultivation in BMMY medium was performed under standard conditions for shaking flask expression (Life Technologies-Invitrogen). Here, highest activities were measured with the Mut^s strain, whereas the expression with the Mut$^+$ phenotype showed lower yields and stagnated (Figure 2A and 2B). The cultivation in FM22 medium was performed under conditions simulating the fermentation process, starting with a glycerol batch followed with the methanol induction phase after glycerol depletion [16]. Interestingly, both Mut phenotypes showed similar

expression behavior in mineral salt medium. However, despite higher cell densities the specific papain activity in the supernatant was much lower than in the complex medium BMMY (Supplementary Figure 1, Figure 2B and 2D). It is possible that the protein stability was reduced in FM22 medium, or that the cells suffered nutritional shortage under those high cell densities in shaking flasks hindering an efficient protein production. Nevertheless, we could demonstrate propapain expression in FM22 medium. This is of particular interest for the establishment of a bioprocess for large-scale, fermentative papain production. Therefore, mineral salt media like the FM22 medium are more cost efficient and allow a better reproducibility of the process than media containing complex components like yeast extract which can lead to batch-dependant variations. Further experiments will be performed to optimize the expression in FM22 medium and to investigate propapain production in a stirred system under controlled cultivation conditions.

In summary, the expression of papain in *P. pastoris* showed a clear advantage over the *E. coli* system. Although the so far highest reported yield of 400 mg/L of recombinant papain was obtained in *E. coli*, the enzyme was expressed as insoluble protein and accumulated in inclusion bodies [8]. Thus, cell lysis, solubilization and refolding were required. We demonstrated that similar yields of soluble protein (463 mg/L) could be produced in *P. pastoris* and purified in a single step from the culture medium. Only Dufour et al. [13] and Versari et al. [14] have so far used the *P. pastoris* expression system for the recombinant production of a papain enzyme with engineered nitrile hydratase activity. Therefore they used a wild type papain sequence with a single carefully selected, tailor-made mutation at the active site of the enzyme thereby creating a nitrile hydratase. Using this engineered but not codon-optimized sequence Dufour et al. [13] could express only 3-5 mg/L of the proenzyme in *P. pastoris* [13]. Thus, with the expression of a synthetic codon-optimized propapain sequence in *P. pastoris* we obtained the highest papain yields so far described in yeasts.

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