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High level expression of human enteropeptidase light chain in *Pichia pastoris*

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ABSTRACT

Human enterokinase (enteropeptidase, rhEP), a serine protease expressed in the proximal part of the small intestine, converts the inactive form of trypsinogen to active trypsin by endoproteolytic cleavage. The high specificity of the target site makes enterokinase an ideal tool for cleaving fusion proteins at defined cleavage sites. The mature active enzyme is comprised of two disulfide-linked polypeptide chains. The heavy chain anchors the enzyme in the intestinal brush border membrane, whereas the light chain represents the catalytic enzyme subunit. The synthetic gene encoding human enteropeptidase light chain with His-tag added at the C-terminus to facilitate protein purification was cloned into *Pichia pastoris* expression plasmids under the control of an inducible AOX1 or constitutive promoters GAP and AAC. Cultivation media and conditions were optimized as well as isolation and purification of the target protein. Up to 4 mg/L of rhEP was obtained in shake-flask experiments and the expression level of about 60–70 mg/L was achieved when cultivating in lab-scale fermentors. The constitutively expressing strains proved more efficient and less labor-demanding than the inducible ones. The rhEP was immobilized on AV 100 sorbent (Iontosorb) to allow repeated use of enterokinase, showing specific activity of 4 U/mL of wet matrix.

1. Introduction

Enterokinase (synonym: Enteropeptidase, UniProt accession number P98073) EC 3.4.21.9 is a serine peptidase found in the intestinal brush border membrane of the duodenum (Mann and Mann, 1994), within intestinal mucosal fluid and intestinal contents (Fonseca and Light, 1983; Eggermont et al., 1971), that activates trypsinogen by cleavage of the N-terminal peptide following the sequence $(Asp)_4$-Lys (Gasparian et al., 2006). Enterokinase is secreted in the form of an inactive single chain proenteropeptidase from intestinal glands called crypts of Lieberkühn. Duodenase, a trypsin-like protease, activates proenteropeptidase by post-translational cleavage resulting in enteropeptidase heavy and light chains (Zamolodchikova et al., 2000; Hoffmeister et al., 2009). Both chains form a disulfide-linked heterodimer which is composed of the heavy (82–140 kDa) and the light (35–62 kDa) chains in all studied enterokinases (Gasparian et al., 2006).

The heavy chain anchors enterokinase in the intestinal brush border membrane (Huang et al., 2007), strongly influences macromolecular substrate recognition and inhibitor specificity. The light chain is homologous to the trypsin-like serine proteases, and is responsible for the peptidase activity (Lu et al., 1997).

Enterokinase cleaves after lysine residue if the Lys is preceded by four Asp and not followed by a Pro. The high specificity of enterokinase makes it an ideal tool for cleaving fusion proteins at defined cleavage sites. The fusion protein can be purified and cleaved by EK to obtain the target protein (Huang et al., 2007).

The expression and purification of the bovine enteropeptidase catalytic subunit from *Escherichia coli* (Collins-Racie et al., 1995), the methylotrophic yeast *Pichia pastoris* (Vozza et al., 1996), the filamentous fungus *Aspergillus niger* (Svetina et al., 2000), and *Saccharomyces cerevisiae* (Choi et al., 2001) has been described. The light chain of human enteropeptidase fused to the thioredoxin was successfully expressed in *E. coli* (Gasparian et al., 2006, 2003). The largest fraction of rhEP was expressed in the form of insoluble inclusion bodies and then refolded by dialysis method. Only 2% of rhEP recovered its activity in such conditions, nevertheless the specific activity of renatured fraction was five times higher than that of bovine enteropeptidase. There is no mention of rhEP expression in *P. pastoris* in available literature at the moment. In comparison to the bacterium *E. coli*, the heterologous protein expression system

Abbreviations: rhEP, recombinant human enteropeptidase; DOT, dissolved oxygen tension; MD, minimal dextrose medium; MWCO, molar weight cut off; CFU, colony forming unit; ECH, epichlorohydrin; GA, glutaraldehyde; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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in *P. pastoris* offers one substantial advantage: *Pichia* is capable of producing disulfide bonds and modifying the translated proteins by N-linked glycosylation. This means that in cases where disulfide bridges are necessary, *E. coli* might produce a misfolded protein that is usually inactive or insoluble (Schutter et al., 2009).

P. pastoris is capable of metabolizing methanol as its sole carbon source (Ellis et al., 1985). This process runs under control of two genes coding the alcohol oxidase named AOX1 and AOX2, where the AOX1 gene is responsible for the vast majority of alcohol oxidase activity in cell (Higgins and Cregg, 1998). Expression of AOX1 gene is tightly regulated and is induced by methanol to very high levels, typically ≥30% of the total soluble protein in cells grown with methanol as a carbon source. The promoter of AOX1 gene is used to drive the expression of the gene of interest encoding a desired heterologous protein (Ellis et al., 1985; Tschopp et al., 1987).

Efforts have been made to develop new alternative promoters to AOX1 expression system to avoid the use of methanol. The glyceraldehyde-3-phosphate dehydrogenase promoter (GAP) has been used for constitutive expression of many heterologous proteins. The GAP-based expression system is more suitable for large-scale production because the hazard and cost associated with the storage and delivery of large volumes of methanol are eliminated (Zhang et al., 2009). However, not every recombinant protein is suitable to be expressed constitutively, and then an inducible expression system must be chosen.

As a working alternative to the GAP promoter, the ATP–ADP Carrier of the mitochondrial inner membrane (AAC) promoter can be used. It is a strong promoter constitutively expressed in mitochondria with activity up to 10 times higher than other commercial systems of protein expression in yeasts (Stadlmayr et al., 2010, 2008).

In common laboratory practice, the enzymes used in catalytic reaction are not recycled. In case of more expensive enzymes, the reaction setup needs to be altered with regard to efficient utilization of the biocatalyst. This can either be achieved by holding back the enzyme by a membrane using for example an enzyme-membrane reactor (Lütz et al., 2006) or by immobilizing the biocatalyst on or in a carrier material (Mateo et al., 2000a). In both cases, several advantages can be achieved such as stabilization of the biocatalyst, recovery and re-use of enzymes, development of continuously operated enzyme reactors, possibility of multi-enzyme systems, and easy removal of the biocatalyst from the reaction mixture and thus, simplified product purification.

Different immobilization techniques have been developed, including covalent coupling (Hildebrand and Lütz, 2006; Mateo et al., 2000a), enzyme cross-linking molecules (cross-linked enzyme aggregates) (Schoevaart et al., 2004; Bastida et al., 1998; Sheldon et al., 2005), adsorption on a carrier (Kurlemann and Liese, 2004), ionic interactions (Mateo et al., 2000b) and the encapsulation in polymeric gels or membranes (Pierre, 2004; Veum et al., 2004; Temiño et al., 2005). The bovine enterokinase immobilization was already described by (Kubitzki et al., 2008). Immobilized bovine enterokinase retained about 60% of its original activity and it was reused up to 18 times, increasing the total turnover number 419-fold compared to a single application (Kubitzki et al., 2008).

The main aim of the present work was to develop a highly effective expression system of enterokinase production suitable for further scale up. We report high level expression of rhEP in *P. pastoris*, its purification and subsequent immobilization. Several steps of cultivation conditions optimization were performed in order to raise the enterokinase expression level. The process of enterokinase isolation and purification was developed as well. Various methods of human enteropeptidase catalytic subunit immobilization have been tested as well as the impact of immobilization on the enzymatic activity of rhEP.

2. Materials and methods

2.1. Genes, vectors and strains used

Strains used for recombinant expression of rhEP (Table 1), YAE, MAE, YGE and YCE were constructed as follows.

P. pastoris strains Y11430 (wt) and MC100-3 (Mut$^-$ His$^-$) were kindly provided by prof. James Cregg (Keck Graduate Institute of Applied Life Sciences, Claremont, CA, USA). Strain MC100-3 HIS4 was prepared as follows: Mut$^-$ His$^+$ strain was derived from MC100-3. A 2900 bp fragment containing HIS4 gene was obtained from pPIC9K by EcoRV, PstI digestion. The fragment was electroporated into MC100-3, transformants were selected on MD agar plates. The clones were checked for G418 resistance, since the fragment from pPIC9K contains a portion of kanamycin resistance gene which confers resistance to both kanamycin and G418.

The nucleotide sequence of human enteropeptidase light chain (AAC50138.1, bases 785–1019) was optimised for protein expression in *P. pastoris* and synthesized by Genscript (USA) with XhoI site added at 5′end and 6× His tag and NotI site added at 3′end. ADP/ATP carrier promoter (ACP) sequence was deduced from *P. pastoris* genome sequence (FN392322.1, bases 424261–425012) and obtained by PCR reaction with genomic DNA of Y11430 strain as template and flanking primers ACPBglIIL (CCCCAGATCTATAGTAAAGTAGGGTATCTTCAAGTAATAGTATACTAAC) and ACPBstIP (CCCCTTCGAATTTGGAATATTATAGATTTGTAAGAAAGCCTGAACAAG). The primers introduced BglII and BstI restriction sites flanking the sequence, the resulting fragment was 770 bp long. The PCR conditions were as follows: denaturation at 95 °C for 10 s, annealing at 53 °C for 10 s, polymerization with PfuUltra II (Invitrogen) at 72 °C for 25 s; 30 cycles.

The expression plasmids pPZ-EKh and pGZ-EKh were constructed by insertion of XhoI, NotI digested rhEP into pPICZαC or pGAPZαC, respectively, digested by the same enzymes. The resulting plasmids were amplified in *E. coli* DH5α and linearized before electroporation into *P. pastoris* strains, either by SacI (pPZ-EKh) or by AvrII (pGZ-EKh). In order to test the expression under the control of the AAC promoter, the GAP promoter in pGZ-EKh was replaced with the ACP sequence. The GAP promoter was excized from the plasmid through BglII and BstI restriction sites. The promoter sequence ACP was synthesized using the ACPBglIIL and ACPBstIP primers, digested with BglII, BstI and ligated into the pGZ-EKh backbone to replace the original GAP promoter. The resulting recombinant plasmid was named pACZ-EKh. The newly cloned ACP sequence of pACZ-EKh plasmid was verified by DNA sequence analysis. The plasmid was amplified in *E. coli* DH5α and linearized by KpnI prior to electroporation into Y11430.

Electroporation into *E. coli* as well as into *P. pastoris* was done by Gene Pulser Xcell (Biorad, USA), using the following parameters: 2.5 kV, 200 Ω, 25 mF in 2 mm electroporation cuvettes for *E. coli* and 2 kV, 600 Ω, 25 mF in 2 mm electroporation cuvettes for *P. pastoris*. The transformants were grown and selected on Zeocin plates as described in Higgins et al. (1998). Zeocin concentrations varied from 100 to 2000 μg/mL in order to obtain multicopy clones as described in Higgins et al. (1998).

2.2. Cultivation and protein expression

The expression of rhEP in shake flasks was done according to "Pichia expression kit manual" (Invitrogen, 2002; Stratton et al., 1998). BYPS medium (1% yeast extract, 2% pepton and 2% sorbitol in 100 mM K-phosphate buffer pH = 6) was used for shake-flask cultures to allow for comparison of strains with different methanol utilization.

The high-cell density substrate limited cultivations in fermentors were done according to Stratton et al. (1998) with the following