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Heterologous expression and pro-peptide supported refolding of the high specific endopeptidase Lys-C

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ABSTRACT

The high specific lysyl endopeptidase (Lys-C; EC 3.4.21.50) is often used for the initial fragmentation of polypeptide chains during protein sequence analysis. However, due to its specificity it could be a useful tool for the production of tailor-made protein hydrolysates with for example bioactive or techno functional properties. Up to now, the high price makes this application nearly impossible. In this work, the increased expression for *Escherichia coli* optimized Lys-C was investigated. The cloned sequence had a short artificial N-terminal pro-peptide (MGSK). The expression of MGSK-Lys-C was tested using three expression vectors and five *E. coli* host strains. The highest expression rate was obtained for the expression system consisting of the host strain *E. coli* JM109 and the rhamnose inducible expression vector pJOE. A Lys-C activity of 9340 ± 555 $nkat_{Tos\text{-}GPK\text{-}pNA}/L_{culture}$ could be achieved under optimized cultivation conditions after chemical refolding. Furthermore, the influence of the native pre-N-pro peptide of Lys-C from *Lysobacter enzymogenes* ssp. *enzymogenes* ATCC 27796 on Lys-C refolding was investigated. The pre-N-pro peptide was expressed recombinantly in *E. coli* JM109 using the pJOE expression vector. The optimal concentration of the pre-N-pro peptide in the refolding procedure was 100 $\mu g/mL_{refolding\ buffer}$ and the Lys-C activity could be increased to 541,720 $nkat_{Tos\text{-}GPK\text{-}pNA}/L_{culture}$. With the results presented, the expensive lysyl endopeptidase can be produced in high activity and high amounts and the potential of Lys-C for tailor-made protein hydrolysates with bioactive (e.g. antihypertensive) and/or techno functional (e.g. foaming, emulsifying) properties can be investigated in future time studies.

1. Introduction

The lysyl endopeptidase (Lys-C; EC 3.4.21.50) belongs to the peptidase S1 family. Lys-C is specific for the cleavage of peptidyl bonds on the C-terminal side of lysine residues. This kind of specific endopeptidase is produced from several bacterial species, including *Achromobacter lyticus* M497-1 [1], *Pseudomonas aeruginosa* [2] and *Lysobacter* sp. IB-9374 [3].

In the past, the *Achromobacter* protease 1 (AP1) from *A. lyticus* was part of detailed characterization studies [4–8]. This secretory peptidase (UniProt ID: P15636) is synthesized as a zymogen [653 amino acids (AA)], including signal peptide (pre-peptide; 20 AA), pro-peptide (185 AA), mature peptidase (268 AA), and an extension peptide (180 AA), from the N- to C-terminus. The recombinant production of AP1 in *Escherichia coli* has already been described [9]. This peptidase is a useful tool for the initial fragmentation of polypeptide chains in protein sequence analysis [10,11], peptide bond synthesis [12,13] and the processing of fusion proteins to biologically active peptides [14].

Lysobacter, for example, *Lysobacter enzymogenes* (type strain ATCC 29487), are Gram-negative, gliding bacteria [15]. In addition to Lys-C, further endopeptidases from *L. enzymogenes* are alpha-lytic endopeptidase (EC 3.4.21.12) and arginyl endopeptidase (Arg-C). A patent [16] describes the recombinant production of Lys-C from *L. enzymogenes* in *E. coli*. In this patent a synthetic *lys-C* gene, based on the proteolytically active form of Lys-C, which was optimized for the codon usage of *E. coli* was used. The synthetic *lys-C* gene was designed so that the amino acid sequence translated showed an artificial N-terminal pro-peptide (MGSK). The recombinant MGSK-Lys-C formed inclusion bodies, which were solubilized using guanidine hydrochloride and refolded using L-arginine

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as an unspecific folding additive. However, they did not use the N-terminal pro-peptide for the refolding of the recombinant Lys-C. It is reported for many enzymes that the pro-peptide had an important function in protein folding either *in vivo* or *in vitro* [17–20].

The aim of the current project was to establish an *E. coli* based expression system to produce high amounts of recombinant Lys-C. Furthermore, the development of an optimized refolding process for Lys-C using the separately recombinant produced N-terminal pro-peptide of Lys-C in the refolding assay was investigated.

2. Materials and methods

2.1. Chemicals and materials

All chemicals were of analytical grade and purchased from Fluka/Sigma–Aldrich (Taufkirchen, Germany), Carl Roth GmbH (Karlsruhe, Germany) or Applichem (Darmstadt, Germany). Rhamnose monohydrate was obtained from Alfa Aesar (Karlsruhe, Germany) or Genaxxon Bioscience GmbH (Ulm, Germany). The chromogenic peptide Tos-Gly-Pro-Lys-*p*NA (Tos-GPK-*p*NA) was obtained from Bachem (Bubendorf, Switzerland). All enzymes used for recombinant DNA techniques were obtained from New England BioLabs Inc. (NEB; Frankfurt am Main, Germany) or Fermentas GmbH (Thermo Fisher Scientific, Waltham, USA). All primers (Table 1) were synthesized at biomers.net GmbH (Ulm, Germany). Agarose was bought from SERVA Electrophoresis GmbH (Heidelberg, Germany). Kits for molecular biological work were purchased from Fermentas GmbH or Qiagen GmbH (Hilden, Germany). Protein molecular weight marker was from NEB. A MINI-PROTEAN system (Bio-Rad Laboratories GmbH, München, Germany) was used for polyacrylamide gel electrophoresis. The bioreactor cultivations were made with the Multifors system (Infors AG, Bottmingen/Basel, Switzerland). PD-10 columns were obtained from GE Healthcare (München, Germany).

2.2. Baterial strains, plasmids and media

The strains and plasmids used in this study are listed in Table 2. *L. enzymogenes* ssp. *enzymogenes* ATCC 27796 was grown overnight at 37 °C in Medium A containing sucrose 10 g/L, meat extract 10 g/L, casein 5 g/L, potassium di-hydrogen phosphate 0.1 g/L, di-potassium hydrogen phosphate 0.1 g/L, and magnesium sulphate 0.1 g/L. *E. coli* XL-1 Blue was grown in lysogeny broth (LB) containing the appropriate antibiotic [100 µg/mL ampicillin (amp)] required for maintaining the plasmids. *E. coli* XL-1 Blue was used in the plasmid constructions involving the subcloning of DNA fragments. *E. coli* BL21(DE3), *E. coli* BL21(DE3)[pLys-S] and *E. coli* JM109(DE3) carry the gene for T7 RNA polymerase under control of the *lac*Z-promotor and were used combined with the pET-expression vectors. *E. coli* JM109 and *E. coli* DSM 14459 were used for expression using the pJOE-expression vectors, which are under control of the *rha*P_{BAD}-promotor. *E. coli* DSM 14459 is *rha*B-negative and, thus, the metabolization of rhamnose (inductor) is reduced [21]. A defined medium described previously [22] was used for expression experiments. However, we did not vary the concentration of glucose, proline and ammonium sulphate between preculture and main culture. The higher concentration was always used. The induction solution for the pJOE-expression systems was equal to the induction solution described in literature [22]. The pET-expression systems were induced with IPTG (final concentration: 0.5 mM). Some preliminary expression experiments were realized using 2xYT medium (tryptone 16 g/L, yeast extract 10 g/L and sodium chloride 5 g/L) containing 1% (w/v) glucose (see Section "Expression experiments" for details)

Table 2
Strains and plasmids used in this study.

	Induction	Source or reference
Strains		
Lysobacter enzymogenes ssp. *enzymogenes* ATCC 27796	na	DSMZ[a]
E. coli XL1-Blue	na	Stratagene[b]
E. coli BL21(DE3)	IPTG	Novagen[c]
E. coli BL21(DE3)[pLysS]	IPTG	Novagen[c]
E. coli JM109(DE3)	IPTG	Promega[d]
E. coli JM109	Rhamnose	Promega[d]
E. coli DSM 14459	Rhamnose	DSMZ[a]
	Abbreviation	**Source or reference**
Plasmids		
pET20b	na	Novagen[c]
pET16b	na	Novagen[c]
pJET1.2	na	Fermentas[e]
pJOE2792	na	Krebsfänger et al. [30]
pGA18-*MGSK-lys-C*	pLC-0	GeneArt[f]
pET20b_*MGSK-lys-C*	pLC-1	This study
pET16_ΔHis_*MGSK_lys-C*	pLC-2	This study
pJOE_*MGSK-lys-C*	pLC-3	This study
pET16_*MGSK-lys-C*	pLC-4	This study
pET20b_*pre-N-pro*	pNP-1	This study
pJOE_*pre-N-pro*	pNP-2	This study
pJOE_Δpre_*N-pro*	pNP-3	This study
pJET_*FLG-lys-C*	pFLC-1	This study

na: not applicable.
[a] Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.
[b] Agilent Technologies, Santa Clara, USA.
[c] Merck, Darmstadt, Germany.
[d] Promega Corporation, Madison, Germany.
[e] Thermo Fisher Scientific, Waltham, USA.
[f] Life Technology, Darmstadt, Germany.

Table 1
Sequences of the primers used in this study.

Primer	Sequence (5′ → 3′)	Construction of plasmid[a]
P 1_for	GGGAATTCCATATGGGTTCTAAAGGTGTTTCCGGTTCC	pLC-3
P 2_rev	CGCGGATCCTTAAACCGGCGGGGTACCGGTG	
P 3_for	CGGGATCCATATGAAACGCATTTGTGGTTCC	pNP-1
P 4_rev	CGGGATCCTCACTTCTCGCCGCTGGCCG	
P 5_for	CGGGATCCATATGAAACGCATTTGTGGTTCC	pNP-2
P 6_rev	CGGGATCCTCACTTCTCGCCGCTGGCCG	
P 7_for	CAATCATATGGCGCCGGCCTCGCGCCCG	pNP-3
P 8_rev	CTATGGATCCTCATTACTTCTCGCCGCTGGCCGCG	
P 9_for	CATATGAAACGCATTTGTGGTTCCCTG	pFLC-1
P 10_rev	GGATCCTCAGAAGGTGATGCTCCACTTGTC	

for: denotes forward primers (underlined: *Nde*I restriction site).
rev: denotes reverse primers (underlined: *Bam*HI restriction site).
[a] See Table 2 for details.