REFOLDING & PURIFICATION OF THE CATALYTIC SUBUNIT FROM BOVINE ENTEROKINASE EXPRESSED IN *E. COLI*

LAB REPORT

BY

WOLFGANG SKALA

STRUCTURAL BIOLOGY GROUP
DEPARTMENT OF MOLECULAR BIOLOGY
UNIVERSITY OF SALZBURG
BILLROTHSTRAẞE 11
5020 SALZBURG, AUSTRIA

MARCH 2012

1 INTRODUCTION

ENTEROKINASE (enteropeptidase, EC 3.4.21.9, MEROPS identifier S01.156) is a 115 kDa serine protease found in the apical membrane of duodenal enterocytes. It removes the *N*-terminal propeptide from trypsinogen, $\cdots$ DDDDK↓I$\cdots$, thus releasing active trypsin[1]. Congenital enterokinase deficiency leads to severe intestinal malabsorption, which becomes manifest in diarrhea, failure to thrive and general edema[2].

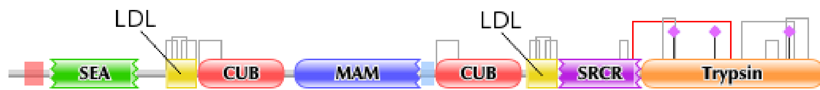


Figure 1: *Enterokinase domains and disulfide bridges.*

Highly similar enterokinase sequences have been isolated from several vertebrate species[3]. In its zymogen form, the holotype from *Bos taurus* (UniProt accession P98 072) comprises a single chain with 1 035 residues (Fig. 1). An as yet uncharacterized protease cleaves this precursor between Lys800 (i. e., Lys15 according to the chymotrypsinogen numbering scheme[4]) and Ile801 (Ile16), yielding an *N*-terminal heavy chain and a *C*-terminal light chain, which remain linked by a disulfide bond (red in Fig. 1). The heavy chain contains two CUB domains, two LDL-receptor class A domains, one MAM domain, one SEA domain and one SRCR domain[5]. It anchors the protein in the cell membrane and provides substrate-binding sites that increase the light chain's activity towards trypsinogen[6].

The light chain is the only domain of enterokinase whose structure has been determined by X-ray crystallography[7]. Its structure belongs to the chymotrypsin fold, featuring two six-stranded β-barrels ($\beta1$ to $\beta6$ and $\beta7$ to $\beta12$, respectively) and three α-helices ($\alpha1$ to $\alpha3$, see Fig. 2a). In addition, enterokinase contains a short β-strand ($\beta1$') adjacent to $\beta8$, and a 3_{10}-helix ($3_{10}1$') between $\alpha1$ and $\beta4$. Five disulfide bonds stabilize the structure. In mature enterokinase, the Cys1–Cys122 bond links the light chain to its "propeptide", i. e. the heavy chain. Asp102, His57 and Ser195 form the catalytic triad, the amide nitrogens of Gly193 and Ser195 serve as oxyanion hole.

In the crystal structure, the covalently bound inhibitor VD_4K-CMK reveals the enterokinase residues that contribute to its substrate specificity. Asp189 neutralizes the ε-amino group of P1-lysine at the bottom of the S1 pocket. In addition, this group forms hydrogen bonds with the hydroxyl and amide oxygen of Ser190 and with two water molecules (Fig. 2b). Both P2-aspartate Oδ interact with Lys99 Nζ, as does one Oδ of P4-aspartate. One P3-aspartate Oδ forms a hydrogen bond with the phenol oxygen of Tyr174 (Fig. 2c)[7].

Due to the clean cuts it makes after the non-primed site recognition sequence, enterokinase is frequently used in biotechnology, for example,

CUB *Complement C1r/C1s, Uegf, Bmp1*

LDL *Low-density lipoprotein*

MAM *Meprin, A5, tyrosine phosphatase μ*

SEA *Sea urchin sperm protein, enterokinase, agrin*

SRCR *Scavenger receptor cysteine-rich*

VD_4K-CMK *Val-Asp-Asp-Asp-Asp-Lys chloromethyl ketone*

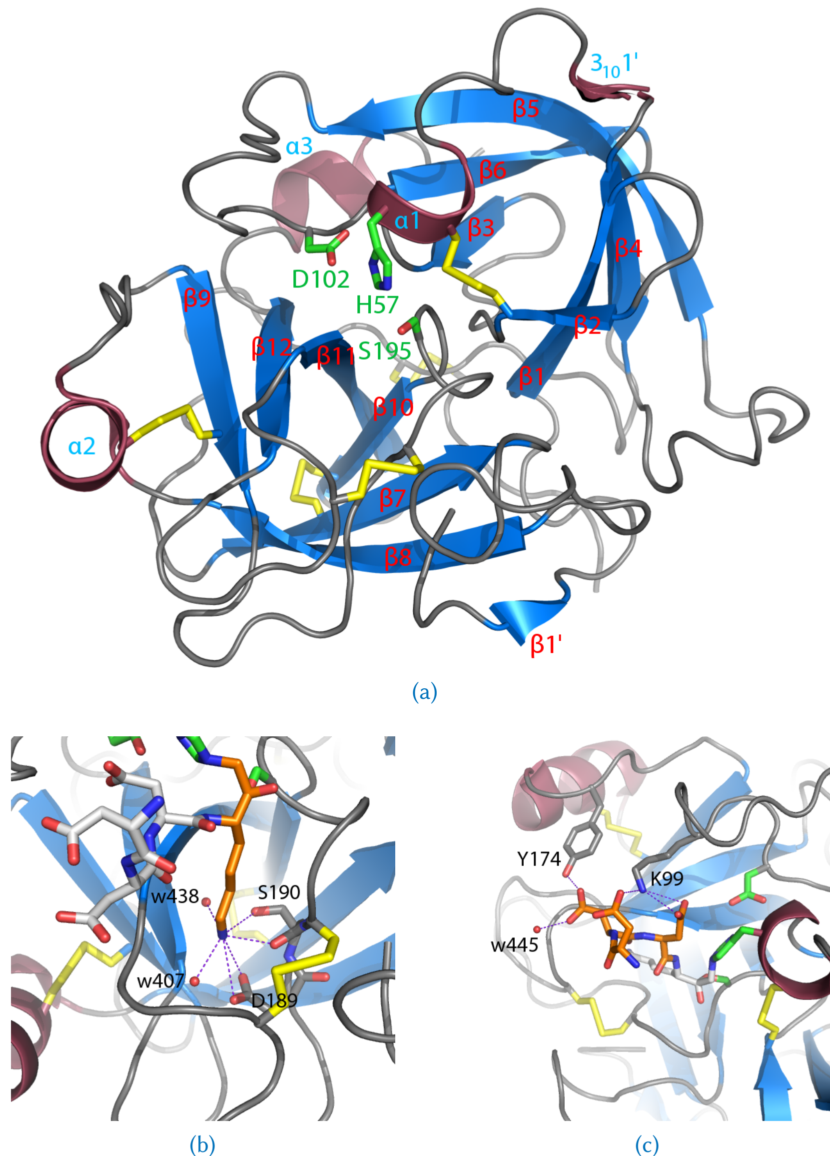


Figure 2: *Crystal structure of the enterokinase light chain.* (a) Overall structure of the protein. Blue (■), α-helices; red (■), β-strands; green (■), active-site residues; yellow (■), disulfide bridges. (b) Interactions between P1-lysine (orange, ■) and residues in the S1 pocket. (c) Interactions between the side chains of the P2–P4 aspartate residues (orange) and their respective binding partners.

to separate an affinity tag from a target protein. Therefore, we set out to develop a cheap, high-yield protocol for producing recombinant enterokinase. Several groups have succeeded in expressing enterokinase recombinantly in *Escherichia coli*: Some of them reported soluble expression[8–11], while others had to refold insoluble protein[7,12]. We failed to reproduce the soluble-expression approach described by Tan *et al.*[11]. However, we were able to adapt a refolding protocol established for other trypsin-like serine proteases. Thus, we successfully refolded and purified a highly active enterokinase triple mutant (P92R C122S E185D) carrying a *C*-terminal pentahistidine tag from bacterial IBs.

IB *Inclusion body*

2 MATERIALS & METHODS

¶ *Materials.* All enzymes used during cloning and the polyacrylamide gel marker were purchased from Fermentas (St. Leon-Rot, Germany). A pET-41a-GST_EK vector encoding Chinese Northern Yellow Bovine enterokinase with an *N*-terminal GST-tag (as described by Tan *et al.*[11]) was kindly provided by Haidong Tan. Bz-PFR-pNA, Suc-AAPR-pNA and Tos-GPR-pNA were obtained from Bachem (Weil am Rhein, Germany), Chromozym X was bought from Roche (Grenzach-Wyhlen, Germany). Recombinant porcine enterokinase was provided by GenScript (Piscataway, NJ, USA). Chemicals were either from Merck (Darmstadt, Germany), AppliChem (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO, USA) of the highest purity available.

¶ *Cloning.* Plasmid pET-41a-EK was prepared from pET-41a-GST_EK and pET-41a-EK_C122S was subsequently obtained from pET-41a-EK by round the horn site-directed mutagenesis. In brief, a part of the respective template vector was amplified by PCR (Mastercycler ep gradient; Eppendorf; Hamburg, Germany) using 5'-phosphorylated primers (Tab. 1a) and the temperature programs shown in Tab. 1b. After the template had been digested by *Dpn*I, amplificates were circularized via blunt end-ligation. Plasmids pET-15b-EK_His5 and pET-15b-EK_C122S_His5 were cloned by (1) amplifying the enterokinase-coding sequences of pET-41a-EK and pET-41a-EK_C122S, respectively (see Tab. 1a and Tab. 1b), and by (2) inserting the amplificates between the *Nco*I and *Xho*I sites of pET-15b. Sequencing confirmed that each clone was correct (Eurofins MWG Operon; Ebersberg, Germany).

¶ *Expression.* Vectors were transformed into *E. coli* BL21(DE3) cells. 600 mL shaker cultures were inoculated 1 : 40 (v/v) from an O/N preculture and incubated at 37 °C and 230 rpm. As soon as the cultures reached an OD_{600} of 1.0, protein expression was induced with 0.5 mM IPTG and continued for 4 h at the same temperature.

¶ *IB Solubilization.* Harvested cells were resuspended 1 : 3 (w/v) in 50 mM Tris *pH* 7.0. After cell lysis by sonication, IBs were washed twice with 50 mM Tris *pH* 7.0, 500 mM NaCl, 20 mM EDTA, 2 % Triton X-100, and once with 50 mM Tris *pH* 7.0, 20 mM EDTA. The resulting IBs were solubilized 1 : 20 (w/v) in 7.5 M guanidine-HCl, 50 mM Tris, 100 mM β-mercaptoethanol. After its *pH* had been adjusted to 3.5–4, the solution was dialyzed against 5 mM citrate *pH* 3.5–4.0. Precipitated protein was collected by centrifugation and solubilized 1 : 10 (w/v) in 7.5 M guanidine-HCl, 50 mM Tris *pH* 4.0–4.5.

GST *Glutathione S-transferase*

Bz-PFR-pNA *Benzoyl-Pro-Phe-Arg-p-nitroanilide*

Suc-AAPR-pNA *Succinyl-Ala-Ala-Pro-Arg-p-nitroanilide*

Tos-GPR-pNA *Tosyl-Gly-Pro-Arg-p-nitroanilide*

PCR *Polymerase chain reaction*

O/N *Over night*

IPTG *Isopropyl β-D-1-thiogalactopyranoside*

EDTA *Ethylenediaminetetraacetic acid*

1	*pET-41a-EK* 5'-TATAGGGGACATATGTATATCTCCTTC-3' 5'-GATGACGACGACAAGATTGTCGG-3'
2	*pET-41a-EK_C122S* 5'-GCTAATAGGCTGTATATAATCTGTGTAG-3' 5'-TTACCAGAAGAAAATCAAGTTTTTC-3'
3	*pET-15b inserts* 5'-ATGCCTCGAGCTAATGATGATGATGATGTAGAAAACTTTGTATCCACTCT-3' 5'-ATGCCCATGGGCCCTATAGATGACGACGACAAGATTG-3'

(a)

	pET-41a-EK		pET-41a-EK_C122S		pET-15b inserts		
	T (°C)	time (s)	T (°C)	time (s)	T (°C)	time (s)	
	95	120	95	120	95	120	
{	95	20	95	20	95	20	}
{	55	20	50–55	20	55–65	20	} 30×
{	72	100	72	80	72	15	}
	72	300	72	300	72	300	
	4	∞	4	∞	4	∞	

(b)

Table 1: *PCR parameters.* (a) Primers and (b) temperature programs for cloning the enterokinase constructs. Primer pair 2 replaces the Cys122 codon TGT with the serine codon AGC. Primer pair 3 adds restriction sites (■/■, the shades of blue indicate the cleavage site) to both termini of the amplificate and four histidine codons to the future 3'-end of the enterokinase-coding sequence. Gray (■), arbitrary overhang; green (■), start codon; red (■), stop codon; yellow (■), bases that align to the template.

¶ *Refolding. In vitro* folding followed a protocol developed by Zögg & Brandstetter[13] and A. Grießner (personal communication). It was initiated by dropwise dilution of the IB solution into the 100-fold volume of a buffer containing 0.5 M arginine, 50 mM Tris *pH* 8.3 at RT, 20 mM $CaCl_2$, 1 mM EDTA, 5 mM cysteine, 0.5 mM cystin. After 72–96 h at 16 °C, the solution was concentrated to the twentieth part of the original volume by tangential flow on wet ice and dialyzed against storage buffer (50 mM Tris *pH* 7.5, 50 mM NaCl).

¶ *Purification.* The enterokinase concentrate was loaded onto a benzamidine sepharose 4 Fast Flow (high sub) column (GE Healthcare; Solingen, Germany). After washing the resin with 50 mM Tris *pH* 7.5, 500 mM NaCl, correctly folded enterokinase was eluted with the same buffer supplemented with 25–100 mM benzamidine. The eluates were concentrated to approximately 0.1 mg mL^{-1} (Amicon Ultra-15 Centrifugal Filter Units; Millipore; Billerica, MA, USA), while the buffer was exchanged to storage buffer containing 10 % (v/v) glycerol. Enterokinase was stored at −80 °C.

The appendix provides a detailed protocol covering all steps from expression to the purified protease.

RT Room temperature

¶ *Kinetics.* Enterokinase activity was measured with 800 ng of the enzyme in a final volume of 100 µL 50 mM Tris *pH* 7.5, 50 mM NaCl. Chromogenic substrates were used at final concentrations ranging from 0.05–40 mM. Time-dependent substrate cleavage was observed by measuring absorbance at 405 nm. Protein concentrations were estimated by absorbance at 280 nm, using extinction coefficients and molecular weights given in Tab. 2. In order to test enterokinase against a protein substrate, pro-KLK4[14] (1 mg mL^{-1}) was incubated with enterokinase for 15 h at RT, and molar ratios were varied. The reaction buffer contained 50 mM Tris *pH* 8.0 and 250 mM NaCl.

Protein	*MW* (kDa)	ε_{280} (M^{-1} cm^{-1})
EK_C122S_His5	26.84	53 900
Porcine enterokinase	26.37	55 390

Table 2: *Parameters for measuring protein concentrations.* Molecular weights and extinction coefficients were calculated by ProtParam (http://expasy.org/tools/protparam.html).

3 RESULTS & DISCUSSION

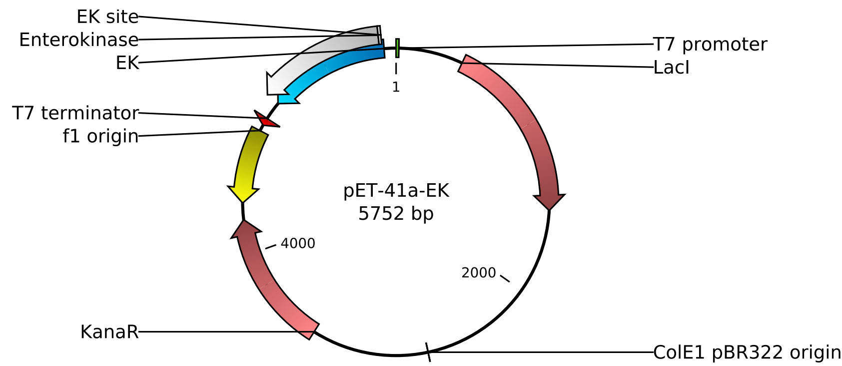
¶ *Construct Design.* Due to its large *N*-terminal GST-tag, the original enterokinase fusion protein[11] resisted effective refolding. Thus, we removed all *N*-terminal tags, which yielded the EK construct (Fig. 3a), a double mutant (P92R E185D) of bovine enterokinase whose propeptide allows autoactivation. We subsequently improved EK in two ways: Firstly, we introduced a third point mutation that changed Cys122 to Ser, which yielded EK_C122S. This step eliminated a surface residue likely to form disulfide bonds with other molecules during *in vitro* folding and consequently improved overall yields. Secondly, we added four histidines to the *C*-terminus of both EK and EK_C122S, thus generating EK_His5 and EK_C122S_His5, respectively (Fig. 3b). The pentahistidine tag of these constructs facilitated purification by IMAC and separation of enterokinase from a protein substrate after its digestion.

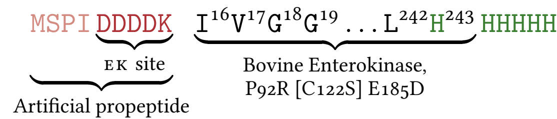
¶ *Expression & IB Solubilization.* The amount of enterokinase steadily increased during expression, yielding a strong band dominating a low background after 4 h (Fig. 4a). Despite intense washes with detergent-containing buffers, most of the target protein remained in the IBs (Fig. 4b). Although minor amounts dissolved in the supernatants, the high purity of enterokinase in solubilized IBs prior to refolding argued for an efficient washing strategy (Fig. 4c).

¶ *In vitro Folding & Purification.* Refolded enterokinase could be separated from incorrectly folded species via its affinity to a benzamidine-coupled resin (Fig. 5a). Since the protein was > 95 % pure after elution from this resin, we refrained from further purification steps. IMAC

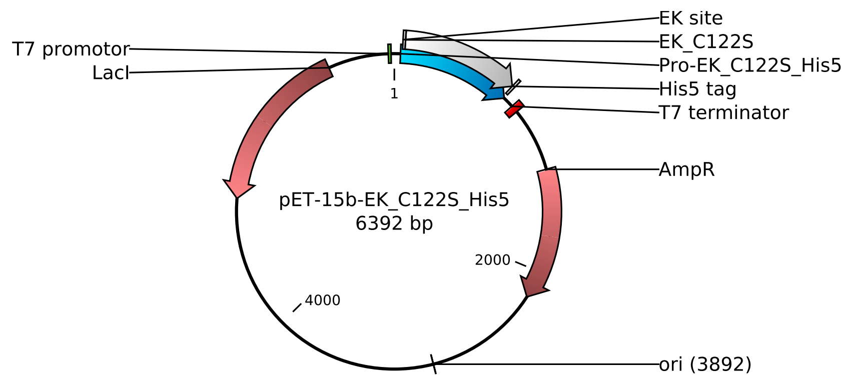
KLK4 *Human kallikrein-related peptidase 4*

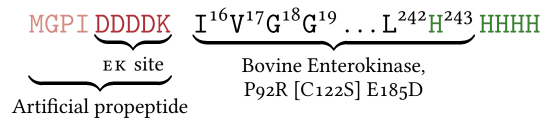
IMAC *Immobilized metal ion affinity chromatography*





(a)





(b)

Figure 3: *Enterokinase constructs.* Features of (a) the pET-41a vectors that encode EK or EK_C122S and of (b) the pET-15b vectors that encode EK_His5 or EK_C122S_His5. Amino acid sequences of these constructs are indicated below each vector.

demonstrated that pentahistidine-tagged constructs were capable of binding to a Nickel column (Fig. 5b). In summary, the yield of properly folded enterokinase was approximately 0.1 % (w/w) of solubilized IBs and in the low mg range per L of bacterial culture.

¶ *Kinetics.* Although enterokinase cleaved a range of chromogenic substrates, it did so with low affinity. For example, we estimated a K_M value of 6.71 ± 0.33 mM for Bz-PFR-pNA and a value of 17.5 ± 1.8 mM for Chromozym X (Fig. 6a and b). In the former case, this is an order of magnitude larger than the value (265 ± 13 μM) reported for human enterokinase against a similar substrate (containing an *N*-terminal ben-

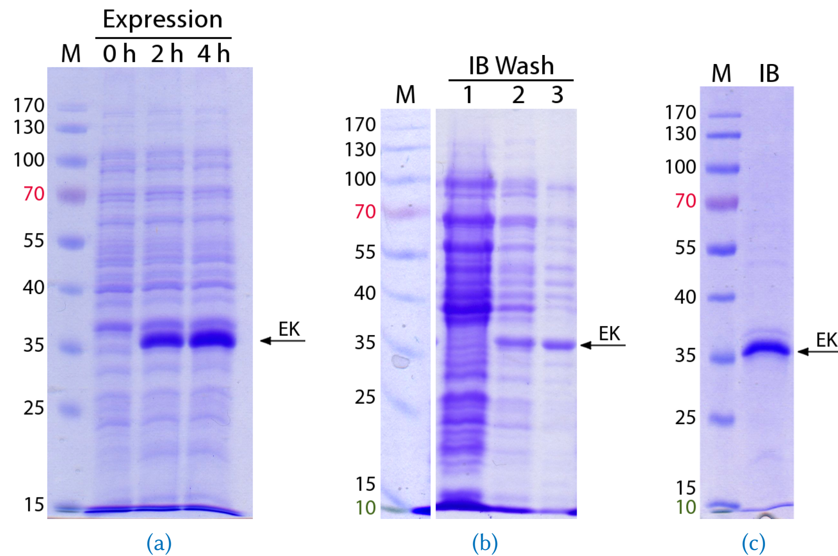


Figure 4: *Preparation of bacterial* IBs. (a) Time course of EK_C122S_His5 expression in *E. coli* BL21(DE3) following induction with 0.5 mM IPTG. (b) Supernatants after washing the IBs with 50 mM Tris *pH* 7.0, 20 mM EDTA (1–3), supplemented with 500 mM NaCl and 2 % Triton X-100 (1 and 2). (c) IBs solubilized in 7.5 M guanidine-HCl, 50 mM Tris *pH* 4.0–4.5. Note that denatured enterokinase runs at a higher-than-native molecular weight (36 vs. 27 kDa). M, marker.

zyloxycarbonyl group instead of a benzoyl group)[15]. However, when we compared EK_C122S_His5 to recombinant porcine enterokinase, the specific activitites of these two proteases were similar in three of four cases (Fig. 6c).

Furthermore, we tested enterokinase against recombinant pro-KLK4, whose artificial propeptide harbored a recognition sequence for the protease. At a molar ratio of 1 : 1 000, enterokinase completely removed the propeptide within 15 h at RT (Fig. 7). These observations established the recombinant enterokinase light chain as an active and specific protease.

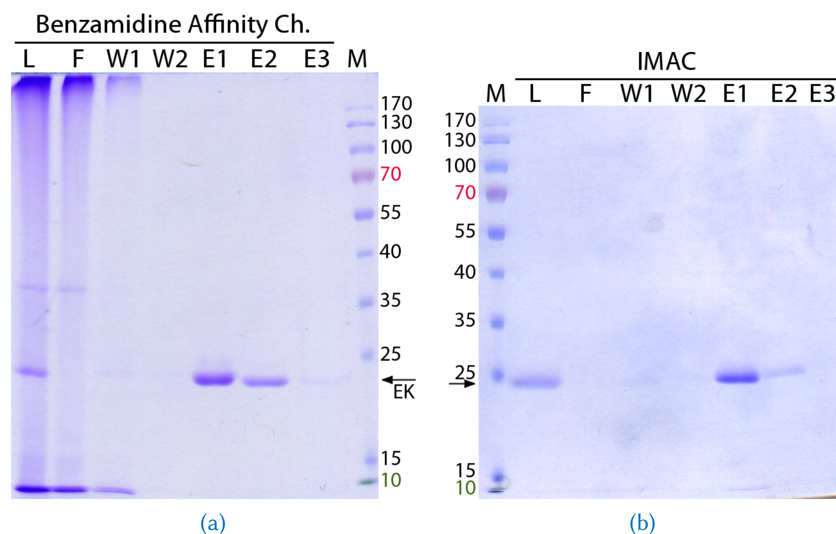


Figure 5: *Purification of enterokinase.* (a) EK_C122S binds to a benzamidine-coupled resin; benzamidine competitively elutes bound protein. M, marker; L, load; F, flow-through; W1 and W2, washes with binding buffer (50 mM Tris *pH* 7.5, 500 mM NaCl); E1–E3, elutions with binding buffer containing 25, 50 and 100 mM benzamidine, respectively. (b) EK_C122S_His5 is captured by immobilized Nickel ions. W1 and W2, washes with binding buffer containing 20 mM imidazole; E1–E3, elutions with binding buffer supplemented with 250 mM imidazole. Note that folded enterokinase runs at a lower-than-native molecular weight (24 vs. 27 kDa).

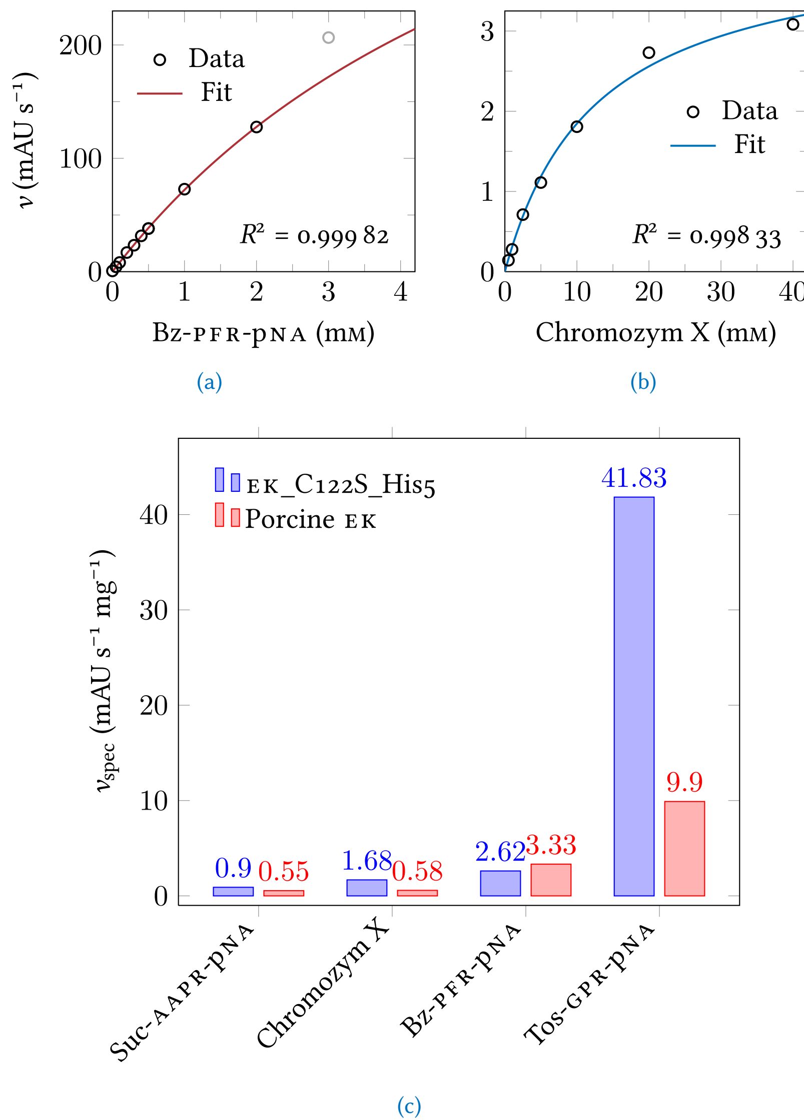


Figure 6: *Enterokinase kinetics.* (a, b) Activity of 800 ng EK_C122S_His5 in 100 µL 50 mM Tris *pH* 7.5, 50 mM NaCl against two peptidic substrates. The gray data point in (a) was excluded from the fit, since Bz-PFR-pNA precipitated at final concentrations larger than 2 mM. (c) Specific activity of EK_C122S_His5 compared to porcine enterokinase. Substrate concentrations were 500 µM. Each value in (a–c) represents the mean of three measurements.

BIBLIOGRAPHY

1 Yamashina, I. (1956). The Action of Enterokinase on Trypsinogen. *Acta Chem. Scand.* 10, 739–743.

2 Holzinger, A. *et al.* (2002). Mutations in the proenteropeptidase gene are the molecular cause of congenital enteropeptidase deficiency. *Am. J. Hum. Genet.* 70(1), 20–25.

3 Kitamoto, Y. *et al.* (1995). cDNA sequence and chromosomal localization of human enterokinase, the proteolytic activator of trypsinogen. *Biochemistry* 34(14), 4 562–4 568.

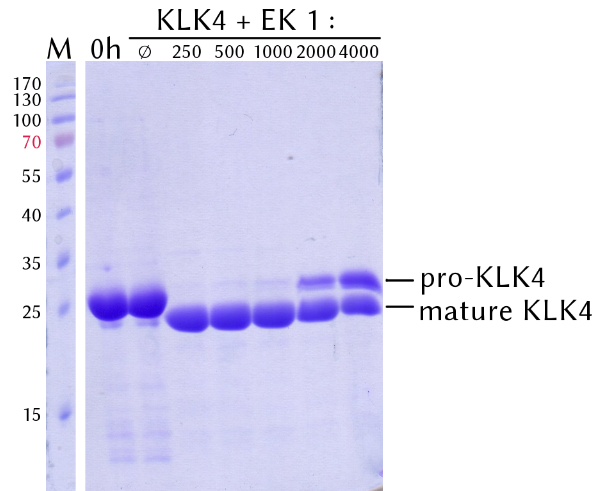


Figure 7: *Enterokinase cleaves pro-KLK4.* 1 mg mL^{-1} pro-KLK4 were incubated with EK_C122S_His5 at the molar ratios indicated for 15 h.

4 Bode, W. *et al.* (1989). The refined 1.9 Å crystal structure of human alpha-thrombin: interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. *EMBO J.* 8(11), 3 467–3 475.

5 Kitamoto, Y. *et al.* (1994). Enterokinase, the initiator of intestinal digestion, is a mosaic protease composed of a distinctive assortment of domains. *Proc. Natl. Acad. Sci. U. S. A.* 91(16), 7 588–7 592.

6 Sadler, J. E. (2004). Enteropeptidase. In: *Handbook of Proteolytic Enzymes.* Vol. 2. Ed. by A. J. Barrett, N. D. Rawlings & J. F. Woessner. Elsevier. Chap. 461, 1 513–1 517.

7 Lu, D. *et al.* (1999). Crystal structure of enteropeptidase light chain complexed with an analog of the trypsinogen activation peptide. *J. Mol. Biol.* 292(2), 361–373.

8 Collins-Racie, L. A. *et al.* (1995). Production of recombinant bovine enterokinase catalytic subunit in *Escherichia coli* using the novel secretory fusion partner DsbA. *Biotechnology (N. Y.)* 13(9), 982–987.

9 Yuan, L.-D. & C. Hua (2002). Expression, purification, and characterization of a biologically active bovine enterokinase catalytic subunit in *Escherichia coli. Protein Expr. Purif.* 25(2), 300–304.

10 Huang, H., Y. Zhao & G. Yi-ru (2004). Prokaryotic expression of Chinese bovine enterokinase catalytic subunit. *Chin. Med. J.* 117(2), 286–290.

11 Tan, H., J. Wang & Z. K. Zhao (2007). Purification and refolding optimization of recombinant bovine enterokinase light chain overexpressed in *Escherichia coli. Protein Expr. Purif.* 56(1), 40–47.

12 Gasparian, M. E. *et al.* (2003). Expression, purification, and characterization of human enteropeptidase catalytic subunit in *Escherichia coli*. *Protein Expr. Purif.* 31(1), 133–139.

13 Zögg, T. & H. Brandstetter (2009). Structural Basis of the Cofactor and Substrate-Assisted Activation of Human Coagulation Factor IXa. *Structure* 17(12), 1 669–1 678.

14 Debela, M. *et al.* (2006). Crystal structures of human tissue kallikrein 4: activity modulation by a specific zinc binding site. *J. Mol. Biol.* 362(5), 1 094–1 107.

15 Gasparian, M. E. *et al.* (2006). Biochemical characterization of human enteropeptidase light chain. *Biochemistry Mosc.* 71(2), 113–119.

ABBREVIATIONS

B

Bz-PFR-pNA	Benzoyl-Pro-Phe-Arg-*p*-nitroanilide

C

CUB	Complement C1r/C1s, Uegf, Bmp1

E

EDTA	Ethylenediaminetetraacetic acid
EK	Bovine enterokinase, P92R E185D
EK_C122S	Bovine enterokinase, P92R C122S E185D
EK_C122S_His5	Bovine enterokinase, P92R C122S E185D, His_5-tag
EK_His5	Bovine enterokinase, P92R E185D, His_5-tag

G

GST	Glutathione *S*-transferase

I

IB	Inclusion body
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl *β*-D-1-thiogalactopyranoside

K

KLK4	Human kallikrein-related peptidase 4

L

LDL	Low-density lipoprotein

M

MAM	Meprin, A5, tyrosine phosphatase μ

O

O/N	Over night

P

PCR	Polymerase chain reaction
pET-15b	Plasmid
pET-15b-EK_C122S_His5	Vector
pET-15b-EK_His5	Vector
pET-41a-EK_C122S	Vector
pET-41a-EK	Vector
pET-41a-GST_EK	Vector

R

RT	Room temperature

S

SEA	Sea urchin sperm protein, enterokinase, agrin
SRCR	Scavenger receptor cysteine-rich
Suc-AAPR-pNA	Succinyl-Ala-Ala-Pro-Arg-*p*-nitroanilide

T

Tos-GPR-pNA	Tosyl-Gly-Pro-Arg-*p*-nitroanilide

V

VD_4K-CMK	Val-Asp-Asp-Asp-Asp-Lys chloromethyl ketone

APPENDIX

DETAILED PROTOCOL

A.1 *Buffers*

¶ LB *Amp Medium.*

Tryptone	10 g L^{-1}
Yeast extract	5 g L^{-1}
NaCl	5 g L^{-1}
Ampicillin	100 µg mL^{-1}
pH	7.0 ± 0.2

¶ *Suspension Buffer I.*

Tris	50 mM	3.03 g (for 500 mL)
pH	7.0	

¶ *Suspension Buffer II.* Prepare as 3× concentrate:

Tris	50 mM	3.03 g (for 500 mL)
NaCl	1.5 M	43.83 g
EDTA	60 mM	8.77 g
Triton X-100	6 %	30 mL
pH	7.0	

Note: Instead of EDTA, you may add 11.17 g Titriplex III (dissolves faster)

¶ *Suspension Buffer III.*

Tris	50 mM	3.03 g (for 500 mL)
EDTA	20 mM	2.92 g
pH	7.0	

Note: Instead of EDTA, you may add 3.72 g Titriplex III (dissolves faster)

¶ *Solubilization Buffer I.*

Guanidine-HCl	7.5 M	716.48 g L^{-1}
Tris	50 mM	6.06 g L^{-1}
β-Mercaptoethanol	100 mM	6.97 mL L^{-1}
pH (native)	≈ 9	

¶ *Citrate Buffer.*

Citrate	5 mM	4.20 g (for 4 L)
pH	3.5–4.0	

¶ *Solubilization Buffer II.*

Guanidine-HCl	7.5 M	358.24 g (for 500 mL)
Tris	50 mM	3.03 g
pH	4.0–4.5	

¶ *Refolding Buffer.*

Arginine	0.5 M	348.4 g (for 4 L)
Tris	50 mM	24.24 g
$CaCl_2$	20 mM	11.80 g
EDTA	1 mM	1.16 g
Cysteine-HCl	5 mM	3.52 g
Cystine	0.5 mM	0.48 g
pH	8.3 at RT	

Note: Dissolve cystine in 5 mL 2 M HCl

¶ *Storage Buffer.*

Tris	50 mM	24.24 g (for 4 L)
NaCl	50 mM	11.68 g
pH	7.5	

¶ *High-NaCl Buffer.*

Tris	50 mM	3.03 g (for 100 mL)
NaCl	5 M	29.22 g
pH	7.5	

¶ *Binding Buffer.*

Tris	50 mM	3.03 g (for 500 mL)
NaCl	500 mM	14.6 g
pH	7.5	

¶ *Elution Buffer 100.*

Binding Buffer		50 mL
Benzamidine-HCl	100 mM	0.78 g
pH	7.5	

¶ *Cryobuffer.*

Tris	50 mM	3.03 g (for 500 mL)
NaCl	50 mM	1.46 g
Glycerol	10 % (v/v)	50 mL
pH	7.5	

A.2 *Protein Expression*

1 Transform *E. coli* BL21(DE3) cells with pET-15b-EK_C122S_His5.
Use either electrocompetent or chemocompetent cells.

2 Inoculate 200 mL LB Amp medium with a single bacterial colony. Incubate this preculture O/N at 37 ℃ and 230 rpm.
Alternatively, obtain the inoculum from a glycerol stock culture.

3 Inoculate 12 × 600 mL LB Amp medium with 15 mL preculture per flask, i. e. 1 : 40 (v/v). Incubate these main cultures at 37 ℃ and 230 rpm.

4 As soon as OD_{600} reaches 1.0 (after about 3 h), induce enterokinase expression by adding 0.5 mM IPTG to the cultures. Maintain temperature and rpm.

5 After 4 h, harvest cells by centrifugation (15 min at 4 000 *g* and 4 ℃).
One pellet should consist of the cells from three cultures (3 × 600 mL). Typical pellet weights range from 8 to 10 g. Pellets can be stored at −20 ℃.

A.3 *IB Solubilization*

1 Resuspend each pellet in Suspension Buffer I to a final volume of 30 mL.

2 Lyse cells by sonication (3 × 5 min with 50 % duty time and 95 % power, interrupted by at least 5 min chilling on ice).

3 Add 15 mL 3× Suspension Buffer II to each lysate and shake it for 30 min at RT.

4 Spin down particulate matter (18 000 *g*, 20 min, RT) and discard supernatant.

5 Resuspend each pellet in 30 mL 1× Suspension Buffer II.
Add 20 mL dH_2O, followed by 10 mL 3× Suspension Buffer II. Use a Potter homogenizer for efficient resuspending.

6 Spin down IBs (18 000 *g*, 20 min, RT) and discard supernatant.

7 Resuspend each pellet in 30 mL Suspension Buffer III.
Again, use a Potter homogenizer.

8 Spin down IBs (18 000 *g*, 20 min, RT) and discard supernatant.
IBs can be stored at −20 ℃. The total IB weight is about 20 g.

9 Dissolve 1 g IB in 20 mL Solubilization Buffer I, i. e. 1 : 20 (w/v).

10 Stir the solution O/N at RT.

11 Spin down undissolved particles (43 000 *g*, 20 min, RT).

12 Adjust the *pH* of the supernatant to 3.5–4.0.
This step is of paramount importance, since a low *pH* prevents random disulfide bonds from forming prior to refolding!

13 Dialyze the supernatant against Citrate Buffer at RT.
The molecular weight cutoff of the dialysis membrane should be 6–8 kDa. Due to the large supernatant volume (e. g., 400 mL), a dialysis ratio of about 1 : 30 suffices (i. e., 3 × 130 mL supernatant against 3 × 4 L Citrate Buffer). Exchange the buffer twice, which yields exemplary dialysis durations of 4 h–4 h–O/N.

14 Harvest precipitated protein by centrifugation (18 000 *g*, 20 min, RT).
Pellets may be stored at −20 °C. The total pellet weigh should approximate the value from step 8 (typically 20 g).

15 Dissolve 1 g IB in 10 mL Solubilization Buffer II, i. e. 1 : 10 (w/v).
If the solution appears cloudy, spin down particulate matter (18 000 *g*, 20 min, RT). The total volume will be about 200 mL.

A.4 *Refolding*

1 Prepare 4 L cups with Refolding Buffer.

2 Dilute the protein solution dropwise into the Refolding Buffer. The final volume ratio must be at least 1 : 100.
In order to minimize the amount of Refolding Buffer required, apply the solution in two pulses separated by an interval of 24 h. For example, if the IB solution volume is 200 mL, prepare 3 × 4 L Refolding Buffer and dilute 2 × 35 mL IB solution into each cup, with one day between the two pulses. Dropwise dilution is achieved by using a burette (or a 5 mL pipette) and stirring the buffer.

3 Incubate the refolding solution for 3–4 d at 16 °C without stirring.

4 Concentrate 4 L refolding solution to about 200 mL by tangential flow on wet ice (molecular weight cutoff 10 kDa).
How to handle the concentrator: (1) Flush the system with 2 L dH_2O, followed by 500 mL Refolding Buffer (without cysteine/cystine). (2) Concentrate the refolding solution to about 100 mL. (3) Rinse the concentrator with 100 mL Refolding Buffer (without cysteine/cystine), which yields a final concentrate volume of 200 mL. (4) Clean the system with 2 L 0.2 M NaOH for 5 min. (5) Store the tubes in 2 L freshly prepared 0.2 M NaOH (adjust 67.5 g 32 % NaOH to 2 L with dH_2O).

5 Dialyze the concentrate against Storage Buffer at RT.
The molecular weight cutoff of the dialysis membrane should be 6–8 kDa. Due to the large concentrate volume (e. g., 600 mL), a dialysis ratio of about 1 : 20 suffices (i. e., 3 × 200 mL supernatant against 3 × 4 L Storage Buffer). A single buffer exchange leads to dialysis durations of 4 h–O/N or *vice versa*.

6 Spin down precipitate (43 000 *g*, 20 min, RT)

7 Add 3 mM NaN_3 to the supernatant and store it at 4 °C for 3 d to ensure complete autoactivation.

A.5 *Purification*

1 Add 20 mL High-NaCl Buffer to 200 mL enterokinase.

This step increases the NaCl concentration to about 500 mM, which prevents unspecific interactions between impurities and the resin.

2 Incubate with 6 mL benzamidine sepharose 4 Fast Flow (high sub) resin (equilibrated with Binding Buffer) for 30 min at RT.

3 Collect the flow-through.

4 Wash the resin with 40 mL Binding Buffer.

5 Elute bound enterokinase with 15 mL Elution Buffer 25, 50 and 100.

Elution Buffer 25 contains 25 mM benzamidine &c.

6 If SDS-PAGE indicates residual enterokinase in the flow-through, repeat steps 2 to 5 (load the flow-through).

7 Combine all eluates, concentrate them to 80–100 µg mL^{-1} and exchange the buffer to Cryobuffer.

For example, three purification batches will yield about 135 mL combined eluate. (1) Concentrate this volume to 3 mL using two Amicon Ultra-15 Centrifugal Filter Units with a molecular weight cutoff of 10 kDa. (2) Dilute the concentrate tenfold with Storage Buffer and concentrate it again to 3 mL. (3) Repeat step (2) twice. (4) Dilute the final concentrate to 80–100 µg mL^{-1} with Cryobuffer.

8 Store enterokinase at −80 °C.

9 Happy digesting! :)