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# Cloning, expression and purification of human and bovine Enterokinase light chain with Cherry tag and their activity comparison

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ABSTRACT

Enterokinase (E.C 3.4.21.9) is a serine protease, known for its specific cleavage after Asp-Asp-Asp-Asp- Lys .Enterokinase (EK) is a widely used tool for in vitro cleavage of recombinant fusion proteins produced by bacterial expression system. So far EK produced in bacterial system mainly expressing as inactive inclusion bodies, which requires an extensive denature and refolding process with low active protein. cDNA encoding the Catalytic Subunit of human Enterokinas was isolated from human dedunum complimentary DNA (cDNA) library (Gene bank accession No. **KF688184**). cDNA Encoding the Catalytic Subunit of Bovine EnterokinasBovine isolated from a Bos taurus indicus (Gene bank accession No. **KC756844**) both cDNA were cloned in psCherry1 vector between EcoR1, Xho1 restriction sites, fused to cherry tag. Human and bovine enterokinase fused with Cherry tag in pS cherry1 expression vectors were expressed in *E. Coli* Rosetta strain. Expressed human and bovine enterokinase light chains with cherry tag, most of the product existed in soluble form. After purification and dialysis auto cleave was observed.14mg/Liter pure active bovine enterokinase light chain protein obtained, without involving any denaturing and refolding process. Likewise 16mg/Liter human enterokinase light chain was purified. Recombinant bovine enterokinase light chain showing $K_m$ value 0.83± 0.03 mM and $K_{cat}$ 25 $Sec^{-1}$ for its speific fluorometric substrate Gly-$(Asp)_4$- Lys-β-naphthylamide. In similar experimental conditions recombinant humane enterokinase light chain showing $K_m$ value 0.085± 0.001 mM and $K_{cat}$ 118 $Sec^{-1}$.

**Key words:** Enterokinase; Cherry tag; Gly-$(Asp)_4$- Lys-β-naphthylamide; fusion protein; cDNA

Enterokinase (EC 3.4.21.9) is a serine protease, responsible for the conversion of the inactive trypsinigen to its active form trypsin[12,21]. The native enzyme consists of a heavy chain (115 kDa) and a catalytic light chain (35 kDa) connected by a disulfide bond[11]. The enzyme possesses a high specificity for the sequence of $(Asp)_4$-Lys[16] and allows release of the carboxyl-terminal fusion partner without leaving unwanted amino acid residues on their amino-termini[10]. The fusion protein as a tool for recombinant protein production is well known. The enterokinase has been shown to have a wide utility in cleaving fusion protein. The enzyme is predominantly suitable for this role because it's able to cleave the fusion protein at wide pH values, ranging from 4.5 to 9.5 at temperatures ranging from 4 to 45°C, and in the presence of various detergents and denaturants[20]. Therefore, Enterokinase (EK) is considered a powerful tool widely applicable in biotechnology[2,6,17-19].

*E. coli* is the first choice for recombinant protein production because of its high level expression and cost effective production. Previous enterokinase expression in *E. coli* was either without any tag[11] or fused with different fusion partners like, trx[7], Dsba[4], and glutathione-S-transferase[13], mostly expressing fusion protein as inclusion bodies, which requires an extensive refolding process with low yield[19], When using the Cherry express vectors (pSCherry1 for N-terminal cherry tag or pSCherry3 for C-terminal cherry tag), the gene of interest is fused to a small sequence encoding a red polypeptide (heme binding part of cytochrome, 11 kDa) providing a visual aid for estimating expression level and solubility. The tag itself being highly soluble, can increase the solubility of the target protein. The red color also constitutes a visual marker throughout protein purification: it is easy to visualize binding of the protein to the column (affinity or ion exchange) and to verify the absence of remaining protein of interest in the effluent[11].

In the present paper we report the fusion of human EK light chain and bovine EK light chain gene with Cherry tag by cloning in ps Cherry1 vector and expressed as soluble form.

*Reagents :*

pS Cherry1 vector was procured from Eurogenta, human duodenum total RNA from Agilent technologies, Restriction enzymes, DNA &protein ladders, and Dioxane free IPTG were from Fermentas, PCR machine from Eppendorf,. Spectra max M5 from Molecular devices, LB Broth, LB Agar, Terrific Broth from Himedia, Hi Fidelity phusion polymerase from finnizymes, Ni-NTA columns from G.E health care, GD4K– β-naphthylamide (GD4K-na)& β-Naphthylamine from sigma, primers synthised by sigma-Aldrich India&Eurofin genomics India, Half area 96 well Black plates from Costar, Chemicals from sigma, mp biomedical and USB, DNA sequencing was done at Eurofins genomics (MWG biotech) India.

*Construction of expression vectors :*

Bovine Enterokinase light chain ($bEK_L$) was amplified by PCR. The vector containing isolated gene, was used as a DNA template. Forward primer designed with Eco R1 Restriction site followed by EK recognition sequence G(D)4K. Reverse primer designed with Xho1

restriction site and without stop codon, which facilitates tag our protein with 6His at c-terminal. Amplified PCR product digested with Eco R1, Xho1 restriction enzymes and cloned between these two restriction sites of psherry1 multiple cloning sites (See Fig. 1).

Human duodenum cDNA library was prepared from human duodenum total RNA by using cDNA synthesis kit. Human duodenum cDNA library was used as a template to amplify human EK light chain. Primer design and cloning strategy (See Fig. 2) remains same as explained for Bovine EK. For primers sequence see Table-1.

*Expression of fusion proteins :*

Construct was first transformed in XL10 Gold competent cells. Plasmid was isolated and after Sequence and reading frame confirmation, transformed into *E. coli* Rosetta expression stain. Transformation mix was plated on LB agar plate containing ampicillin and Chloramphenicol antibiotics. A culture from a single colony was grown at 37°C overnight. 2% over night culture inoculated into 100ml fresh LB Broth/Terrific Broth containing antibiotics ampicillin (100 µg/ml) and Chloramphenicol (34µg/ml), then grown at 37°C until O.D reaches to 0.5-0.6. IPTG was added to a ûnal concentration of 1mM and the culture grew at 30°C for 5 h. The cell pellet was harvested by centrifugation at 6000 rpm for 10min.

*Purification :*

Cell pellet was re-suspended in 4 ml Bacteterial protein extraction reagent B-PER (Thermo fisher scientific product) and to this 2mg/ml lysozyme was added mixed with pipette then incubated for 30 min on ice. The supernatant and pellet fractions were separated by centrifugation at 12,000 rpm, at 4°C for 15min. Supernatant collected in separate tube and pellet dissolved in 500µl of 2Molar urea and 8Molar urea separately.

Lysate was loaded on Ni-NTA columns. Columns were washed twice with wash buffer(50 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4) and protein eluted with elution buffer (50 mM sodium phosphate, 300 mM NaCl, 500 mM imidazole, pH 7.4)500mM [His SpinTrap Product booklet[8], Handbooks from GE Healthcare[1]].

*SDS–PAGE :*

Total cellular protein or the supernatant and the lysate pellet fractions corresponding to 1 O.D were analyzed by SDS–PAGE with 5% stacking gel and 12% separating gel. Samples were treated by heating at 95°C or 60°C for 10 min in 5x SDS sample loading buffer before loading on gels. SDS–PAGE gels were stained with Coomassie blue R250. Protein content in the supernatant and the lysate were compared.

*Auto cleavage of Fusion protein:*

Eluted protein dialysis and concentration was done in 50mM Tris-Cl buffer using Millipore 3 kDa cutoff, 50ml centricons.After concentration of protein, 2mM $CaCl_2$ was added and incubated for 24 hrs. at room temperature.

*Enzyme Kinetics :*

Enterokinase activity and kinetic parameters for cleavage of the fluorogenic Enterokinase substrate GD4K-na were determined, as

described earlier[5]. Assay was performed by adding $rEK_L$ (2.5nM) to substrate solution 50µl containing 0.02- 1mM GD4k-na, 25mM Tris-Cl, 10mM $CaCl_2$, 10%DMSO, pH 8.0, and enzyme activity was measured by increase of fluorescence over 10 min in spectromax M5 instrument (excitation at 337nm and emission at 420nm) Values for $K_m$, $K_{cat}$ were obtained by directly fitting to the Michelis-Menten equation by nonlinear regression, using Graph Pad Prism5 software.

*Protein determination :*

Protein concentration was measured by bi-Cinconic acid method with bovine serum albumin as reference standard[12].

Human Enterokinase light chain sequence exactly matches with sequence reported on gene bank accession number. NM_002772.2, but Bos tarus indicus enterokinase sequence varies one amino acid change at position P121S compared to gene bank accession number L19663. However this mutation doesn't affect its enzyme activity and it exhibits similar activity compared to reference enzyme from Sigma.

Enterokinase has 9 cyctein amino acids and when expressed in *E. coli*, without any tag or with various solubility increasing tags like Trx, GST, DsBA it mainly expresses as inclusion body and in the present study we expressed Enterokinase tagging with small heme protein Cherry tag and found it mostly expressing in soluble form both human as well as bovine EK-Cherry fusion proteins (See Fig. 3&4). Both Ek expression levels are more similar and there could be much similarity between two at amino acid composition.

Due to its small tag auto cleavage was observed without adding any external EK to initiate self cleavage. (Fig. 5) but for efficient auto cleavage addition of $CaCl_2$ to a 2mM and removal of imidazole are required. After auto cleavage Ek light chain was passed by Ni-NTA column and eluted. Buffer exchange was done and finally ~14mg/litre bovine and ~16 mg/litre human enterokinase light chain was obtained

In similar assay conditions bovine EK exhibiting Km Value 0.83 ± 0.03 mM (See Fig. 6), where as Human EK light chain showed 0.085±0.001 (See Fig.7) Km value, which is almost 10 fold more active than bovine Enterokinase (See Table-1). Even though both species of enterokinase have great similarity at amino acid composition but their active sites of differ which could be the reason for their activity differences.

We have observed in our experiments variation in Bovine EK Km value from one assay to another which is more compare to human, which is very consistent at KM value. Human enterokinase more effienciently cleaves small substrate GD4K-na than Bovine. These results confirm the statement by Gasparian[7]. We found that this process for expressing EK is simple and efficient.eventhough cherry tag is small(11kDa), but it significantly makes Enterokinase soluble, where famous soluble fusion partners like Trx,GST, DSB A fail to do. Cherry tag is heat stable it protects fusion protein from heat degradation and purification process doesn't require maintaining cooling process during purification. This process is useful for production of Enterokinase for use in industrial purpose.

Soluble expression of Bovine and

**Figures and tables**

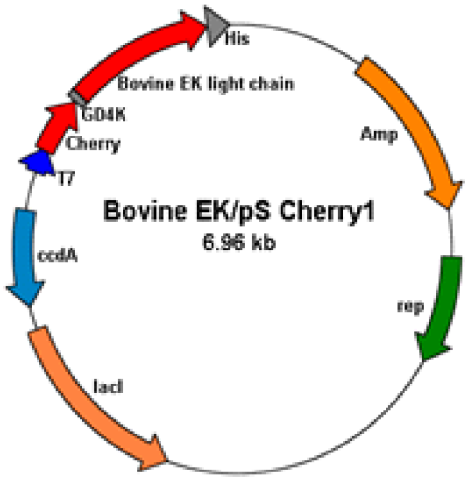


Fig.1. Diagrammatic representation of bovine EK-pS Cherry1 vector construct

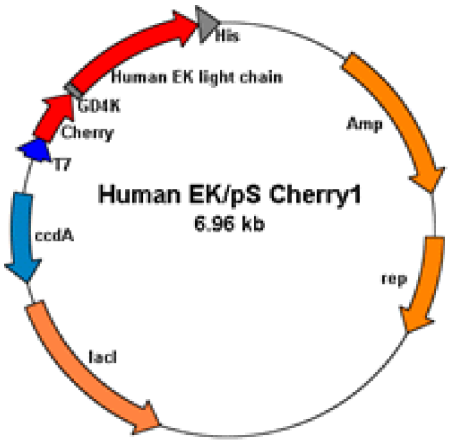


Fig.2. Diagrammatic representation of human EK-pS Cherry1 vector construct

Table-1. Sequence of the Primers

| Primer name | Orientation | Sequence (5'-3') |
|---|---|---|
| Human Ek F.P EcoR1-DDDK | Forward | GGC GAA TTC GAT GAC GAC GAC AAG ATT GTT GGA GGA AGT AAT GCC |
| Bovine Ek F.P EcoR1-DDDK | Forward | GGC GAA TTC GAT GAC GAC GAC AAG ATT GTT GGA GGA AGT G |
| Human/Bovine EK R.P Xhol-No stop | Reverse | GCG GCG CTC GAG ATG TAG AAAACT TTG TAT CC |

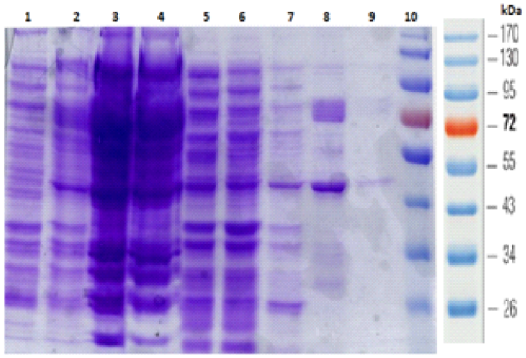


Fig. 3. Bovine Ek/pS Cherry expression profile.Lane1: bEK-psCherry Un Induced, Lane 2: bEk-psCherry Induced, Lane3: pellet dissolved in 2M Urea,Lane4: pellet dissolved in 8M Urea,Lane5: lysate,Lane6: Flow through, Lane 7: wash, Lane 8: Elution 1, Lane 9: Elution 2,Lane10:Prestained MW protein marker

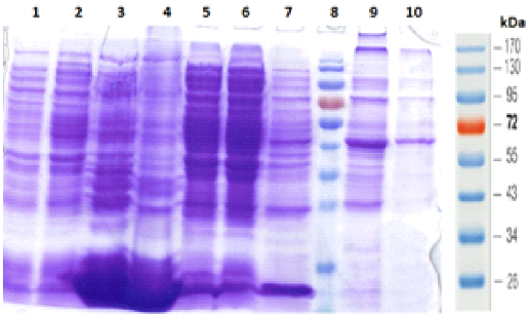


Fig. 4. HumanEk/pS Cherry expression profile.Lane1: hEK-psCherry Un Induced, Lane2: hEk-psCherry Induced ,Lane3: lysate, Lane4: Flow through,Lane5: pellet dissolved in 2M Urea,Lane6: pellet dissolved in 8M Urea,Lane 7:wash,Lane 8:Prestained MW marker,Lane 9: Elution 1, Lane 10:Elution 2

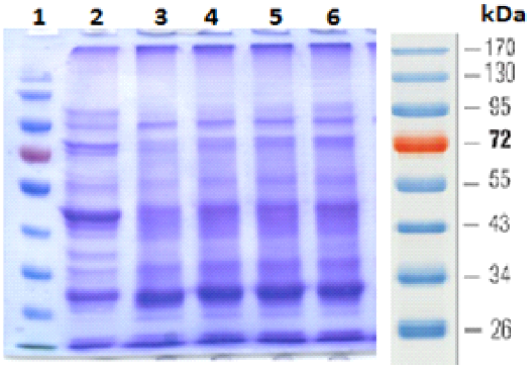


Fig. 5. EK-Cherry auto cleavage. Lane1: Prestained MW marker, Lane2: bEK-Cherry tag protein without $CaCl_2$,Lane3: bEK-Cherry tag protein after 24 hrs at RT (with 2mM$CaCl_2$), Lane4: bEK-Cherry tag protein after 48 hrs at RT(with 2mM$CaCl_2$,Lane5: hEK-Cherry tag protein after 24 hrs at RT (with 2mM$CaCl_2$), Lane6: hEK-Cherry tag protein after 48 hrs at RT(with 2mM$CaCl_2$,

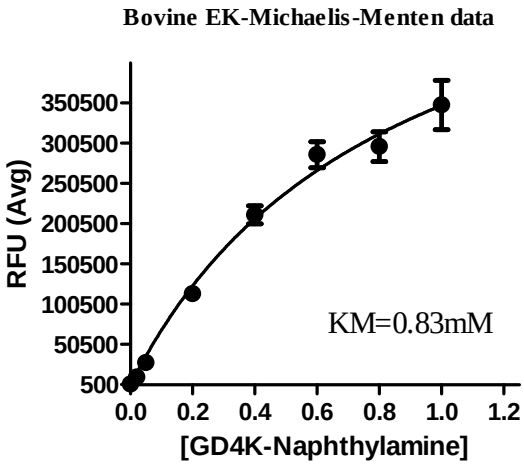


Fig. 6. Initial velocity of bovine Enterokinase for substrate Gly-(Asp)4-Lys-β-naphthylamide

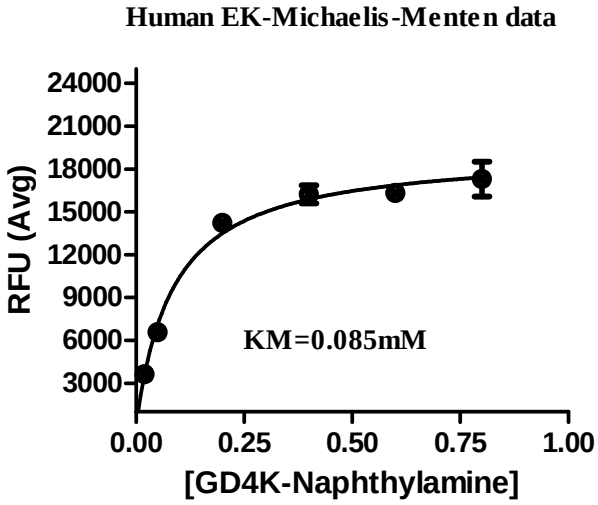


Fig. 7. Initial velocity of humanEnterokinase for substrate Gly-(Asp)4-Lys-β-naphthylamide

Table-2. Comparison of kinetic parameters of human and bovine Enterokinase light chaip

| Enterokinase | GD4K-na(Substrate) | | |
|---|---|---|---|
| | $K_m$(mM) | $K_{cat}$($S^{-1}$) | $K_{cat}$ / $K_m$ ($mM^{-1}$ $S^{-1}$) |
| Human light chain | 0.085±0.001 | 118±3 | 1388 |
| Bovine light chain | 0.83±0.03 | 25±0.2 | 30.12 |

Abbrevations:

PCR-Polymerase chain reaction
cDNA-Complementary DNA
RT PCR-Reverse transcriptase Polymerase chain reaction
SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis Soduin do ducal sulphate poly acrylamide gel electrophoresis
IPTG-isopropylthio-β-galactoside
$rEK_L$ - Recombinant bovine enterokinase light chain
$EK_L$ -Enterokinase light chain
*E.coli- Escherichia coli*
$bEK_L$ bovine enterokinase light chain
mM-Milli molar
nM-Nanomolar
kDa- Kilo Dalton
Trx- Thioredoxin
GDK4–naGly-$(Asp)_4$- Lys-β-naphthylamide
$GD_4K$- Gly-(Asp)4-Lys
µg-Microgram
cm-Centi meter
sec-seconds
min- minutes
LB Broth-Luria-Bertanibroth
LB Agar- Luria-Bertani agar
NaCl-Sodium Chloride
$MgCl_2$ Magnesium Chloride
rpm-rate per minute
DTT- Dithiothreitol
Mg-Milli gram
MW-Molecular weight
bp-base pair
kb-kilo base
Ni-NTA Column-Nickel-nitriloacetic acid column
O.D- optical density
ml-milli liter
PBS- Phosphate-buffered saline
Nm-nano meter
Hrs-Hours

human enterokinase light chain was achieved and with our experiments we conclude that human enterokinase is more active than bovine enterokinase.

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