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Short Communication

Expression and purification of soluble and active human enterokinase light chain in *Escherichia coli*

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ABSTRACT

Human enterokinase light chain (hEK_L) specifically cleaves the sequence $(Asp)_4$-Lys↓X (D_4K), making this a frequently used enzyme for site-specific cleavage of recombinant fusion proteins. However, hEK_L production from *Escherichia coli* is limited due to intramolecular disulphide bonds. Here, we present strategies to obtain soluble and active hEK_L from *E. coli* by expressing the hEK_L variant C112S fused with maltose-binding protein (MBP) through D_4K and molecular chaperons including GroEL/ES. The fusion protein self-cleaved *in vivo,* thereby removing the MBP in the *E. coli* cells. Thus, the self-cleaved hEK_L variant was released into the culture medium. One-step purification using HisTrap™ chromatography purified the hEK_L variant exhibiting an enzymatic activity of 3.1×10^3 U/mL (9.934×10^5 U/mg). The approaches presented here greatly simplify the purification of hEK_L from *E. coli* without requiring refolding processes.

Recombinant fusion technology has been used to enhance the expression level and solubility of target proteins, and to facilitate their purification [1,2]. Proteases including Factor Xa, thrombin, tobacco etch virus (TEV) protease, and enterokinase (EK) are used for the site-specific cleavage of recombinant tags from fusion proteins [3–6]. While Factor Xa, thrombin, and TEV protease cleave inside the recognition site, EK cleaves outside the site, thus it has a proteolytic activity regardless of the P1′ position sequence.

Human EK (hEK) (DDDDK↓, D_4K↓) is produced by cells in the duodenum and intestinal brush-border [7–9]. EK activates trypsin by cleavage of trypsinogen [10–12]. hEK consists of an 86 kDa heavy chain and a 28 kDa light chain that are connected by a single disulphide bond. The heavy chain contains an intestinal brush-border membrane-binding motif. The light chain harbours the classical catalytic triad (chymotrypsin His57, Asp102, and Ser195) with four intramolecular disulphide bonds. The hEK light chain (hEK_L) can cleave the fusion protein to obtain the authentic form of the protein [13]. In addition, hEK_L is an attractive protease for use in protein purification due to its broad range of reaction conditions (pH 4.5–9.5 and temperature 4–45 °C), tolerance against various detergents, and reusability [10,12].

hEK_L has a 10-fold higher catalytic efficiency (k_{cat}/K_M) than bEK_L [14,15]. However, several reports show that hEK_L is expressed in inclusion bodies in *E. coli* [10] that necessitates refolding using dialysis [16–19], dilution [18,20–22], or on-column methods [18,23–25].

In this study, we present strategies to produce active hEK_L in *E. coli* cytoplasm. We report production of soluble, active hEK_L with improved folding efficiency that can be used in-house. To produce active, cytoplasmic hEK_L with the correct disulphide bonds, we constructed hEK_L fused with MBP through the D_4K cleavage site and expressed this in *E. coli* cells expressing chaperone proteins (Fig. 1a). A previous report demonstrated expression of soluble and active MBP-tagged hEK_L [26]. However, we found that MBP-hEK_L was unable to self-cleave, indicating an absence of the enzymatic activity (Figs. S1 and 1b). To test whether removal of MBP could restore the hEK_L activity, an hEK_L variant was constructed by replacing the D_4K with the TEV protease recognition site (ENLYFQ). However, hEK_L obtained by TEV cleavage of MBP-$hEK_L$was still inactive (data not shown). To investigate whether the loss of activity resulted from a limited reduction of disulphide bonds or misfolding, we conducted a refolding process to rearrange disulphide bonds. Detection of self-cleaved forms of refolded hEK_L indicated that the refolded enzyme was partially active (Fig. S2).

Abbreviations: D_4K, $(Aspartic\ Acid)_4$ Lysine; EK, enterokinase; bEKL, bovine enterokinase light chain; hEKL, human enterokinase light chain; IPTG, isopropyl β-D-1-thiogalactopyranoside; MBP, maltose-binding protein; TEV, tobacco etch virus.

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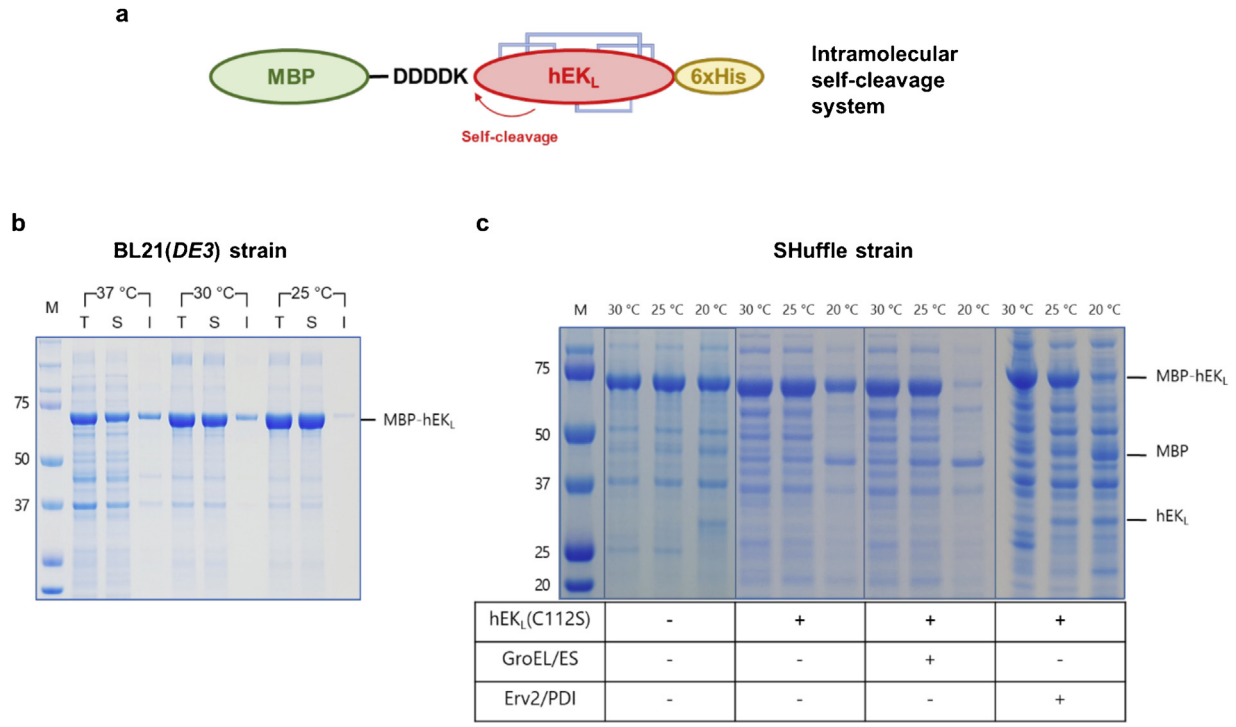

Fig. 1. The expression and activity analysis of hEK$_L$ in flask culture. (a) Construction of MBP-hEK$_L$ fusion connected through the EK cleavage sequence. (b) Expression of MBP-D$_4$K-hEK$_L$ in *E. coli* BL21 (*DE3*) at different temperatures. (c) The expression of hEK$_L$ C112S in *E. coli* SHuffle strain. The blue lane in 1a indicates disulphide bonds. M, Protein marker; I, Insoluble protein; S, soluble protein; T, Total protein.

These results demonstrated that MBP fusion enhances the solubility of hEK$_L$ but does not allow its correct folding. We speculated that hEK$_L$ misfolding might result from incorrect disulphide bonds formed during expression in *E. coli*.

Therefore, to promote the formation of the correct disulphide bonds in *E.coli*-expressed hEK$_L$, we employed three strategies: (i) use of a *trxB*$^{-}$, *gor*$^{-}$, *ahpC***mutant expressing cytoplasmic *DsbC* (SHuffle strain) for oxidative folding, (ii) replacement of the free cysteine with serine (C112S), which bound to heavy chain, to reduce misfolding, and (iii) co-expression of molecular chaperones that isomerize disulphide bonds. First, when the SHuffle strain was used, self-cleaved hEK$_L$ was successfully detected, although at a low level (7.9 % of total MBP-D$_4$K-hEK$_L$), in cells grown at 20 °C (Fig. 1c). Use of the C112S mutated hEK$_L$ dramatically improved the ratio of self-cleaved hEK$_L$ to up to ~49.5 % in cells grown at 20 °C, which may be caused by the reduced mispairing of multiple disulphide bonds [12,27]. Remarkably, fully self-cleaved hEK$_L$ was detected from cell co-expressing GroEL/ES and Erv2/PDI grown at 20 °C. In particular, the activity was slightly higher upon GroEL/ES co-expression. Notably, hEK$_L$ was not visible in the SDS-PAGE gel even when hEK$_L$ activity was observed. However, as shown in Fig. S3, when inactivated hEK$_L$ was produced by TEVp, hEK$_L$ was visible in the SDS-PAGE gel. Therefore, we assumed that the visibility of hEK$_L$ in the SDS-PAGE gel was influenced by its folding.

We further monitored the time profiles for cell growth and enzymatic activity of hEK$_L$ C112S (Fig. 2a and b). After 27.5 h of culture, the cell growth reached the maximum (2.87 OD_{600}) and then sharply decreased. At that time, the hEK$_L$ activity in the soluble fraction reached the maximum value (372 U/mL) and then decreased to ~22 U/mL. In contrast, hEK$_L$ in culture supernatants reached the maximum value (303 U/mL) after 75.5 h of culture. These results indicated that hEKL may be released into the extracellular fraction by autolysis of cell.

We attempted to obtain highly pure hEK$_L$ C112S from culture supernatants. The culture supernatant of *E. coli* SHuffle expressing pET-30a-MBP-D$_4$K-hEK$_L$ C112S and pACYC-GroEL/ES was loaded on the affinity chromatography (HisTrap^{TM}) along with 1 mM DTT to improve the binding efficacy (Fig. 2c). The enzymatic activity was 306 ± 0 U/mL and 3085 ± 43 U/mL before and after purification, respectively (Fig. 2d–g). A previous report [11] showed that a low-yield hEK$_L$ (10 %) can be purified from the culture media of *P. pastoris* using a two-step purification with several pre-treatment steps [11]. However, we could purify hEK$_L$ at high purity (>99 %) and yield (>99 %) using a simplified one-step method. Purified hEK$_L$ C112S had affinity to GD$_4$K-na with K_M = 0.287 ± 0.079 mM, turnover number $K_{cat} = 6.725 \times 10^4 \pm 1.230 \times 10^4$ s^{-1}, and catalytic efficiency $K_M/K_{cat} = 2.385 \times 10^5$ mM^{-1} s^{-1}.

In conclusion, we could purify soluble and active hEK$_L$ at a high yield using an MBP tag, replacing the free cysteine with serine, using *E. coli* strain promoting oxidative folding, co-expressing molecular chaperone that isomerise disulphide bonds, and culturing at low temperature. These findings provide strategies for purification of the complex, multiple disulphide-bonded hEK$_L$ from *E. coli*.

Author contributions

Y.S.K, H. Lee and S.H. Park designed experiments and collected data. Y.K and J.A. supervised the research project and guided the design of experiments. Y.S.K and H.L drafted the manuscript. All authors read the manuscript and agree to submission to Journal of Biotechnology

Data statement

All data reported in the paper are available from the corresponding author upon reasonable request. Materials and

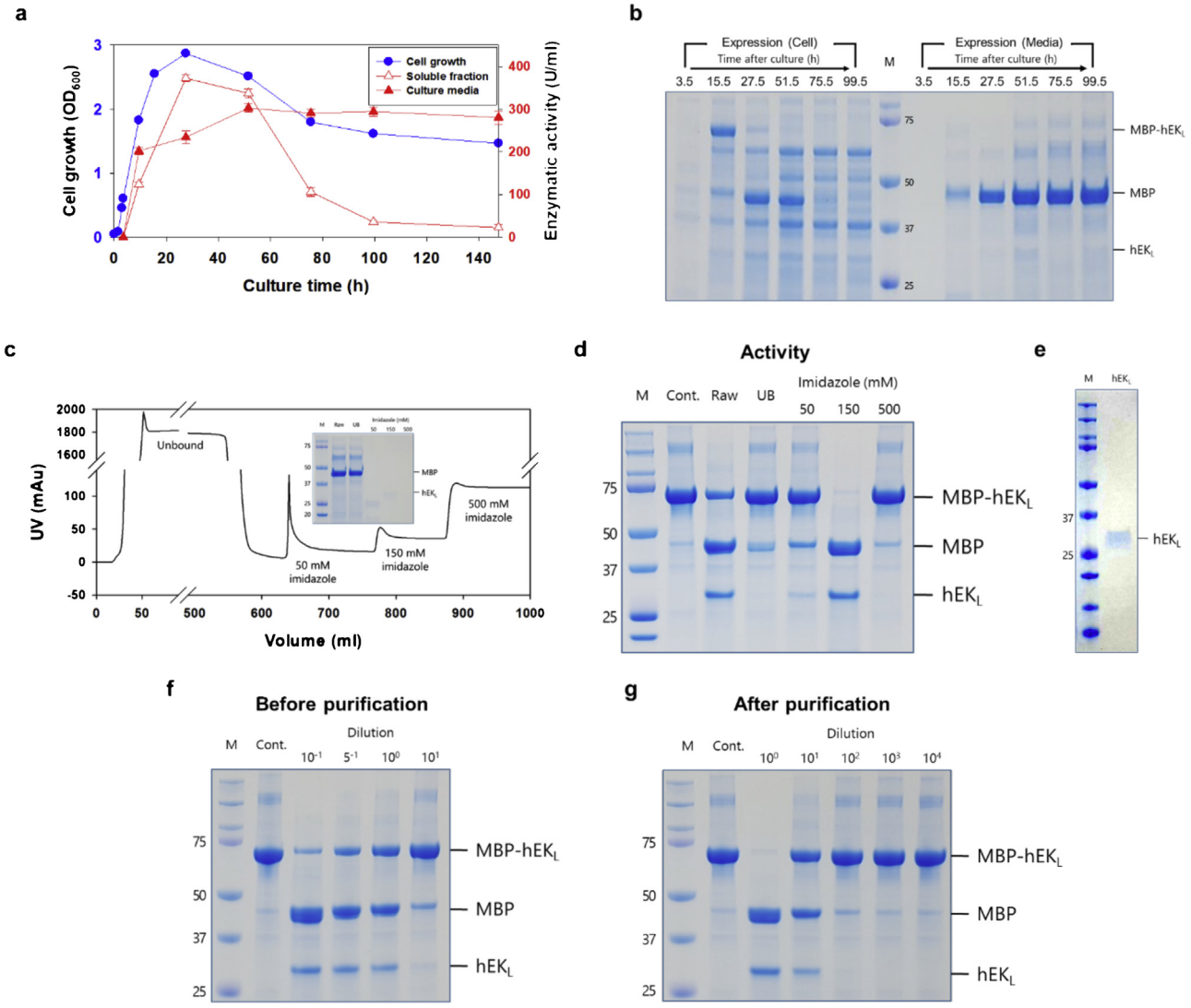

Fig. 2. Expression and purification of hEK_L C112S. (a) Time-profiles of cell growth and activity of hEK_L C112S in flask culture. (b) SDS-PAGE analysis of flask culture samples. (c) Chromatogram of hEK_L C112S purification. The inlet indicates SDS-PAGE of each fraction (raw: load fraction, UB: unbounded fraction). (d) Indirect conformation of enzymatic activity of each eluted fraction. MBP-D_4K-hEK_L (25 μg) was treated with 1 μl of purified hEK_L and incubated at 37 °C for 1 h. (e) SDS-PAGE and western blot of purified hEK_L C112S. Enzymatic activity of hEK_L C112S (f) before purification and (g) after purification. MBP-D_4K-hEK_L (25 μg) was treated with 1 μl of diluted culture supernatant or purified hEK_L and incubated at 37 °C for 1 h. M, Protein marker; Cont., MBP-D_4K-hEK_L.

Methods in this study are described in the Supplementary information.

Declaration of Competing Interest

The authors have no competing interests to declare

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2021.e00626.

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