Short Communication

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

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\textbf{A B S T R A C T}

Human enterokinase light chain (hEK\textsubscript{L}) specifically cleaves the sequence (Asp)\textsubscript{4}–Lys\textsubscript{1}–X (Asp)\textsubscript{K}, making this a frequently used enzyme for site-specific cleavage of recombinant fusion proteins. However, hEK\textsubscript{L} production from \textit{Escherichia coli} is limited due to intramolecular disulphide bonds. Here, we present strategies to obtain soluble and active hEK\textsubscript{L} from \textit{E. coli} by expressing the hEK\textsubscript{L} variant C112S fused with maltose-binding protein (MBP) through D\textsubscript{K}–MBP and molecular chaperons including GroEL/ES. The fusion protein self-cleaved in vivo, thereby removing the MBP in the \textit{E. coli} cells. Thus, the self-cleaved hEK\textsubscript{L} variant was released into the culture medium. One-step purification using Histag\textsuperscript{TM} chromatography purified the hEK\textsubscript{L} variant exhibiting an enzymatic activity of 3.1 \times 10^{3} U/ml (9.934 \times 10^{2} U/mg). The approaches presented here greatly simplify the purification of hEK\textsubscript{L} from \textit{E. coli} without requiring refolding processes.

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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) (DDDKK\textsubscript{1}–D\textsubscript{K}K\textsubscript{1}) 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\textsubscript{L}) can cleave the fusion protein to obtain the authentic form of the protein [13]. In addition, hEK\textsubscript{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\textsubscript{L} has a 10-fold higher catalytic efficiency (k\textsubscript{cat}/K\textsubscript{m}) than hEK\textsubscript{14,15}. However, several reports show that hEK\textsubscript{L} is expressed in inclusion bodies in \textit{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\textsubscript{L} in \textit{E. coli} cytoplasm. We report production of soluble, active hEK\textsubscript{L} with improved folding efficiency that can be used in-house. To produce active, cytoplastic hEK\textsubscript{L} with the correct disulphide bonds, we constructed hEK\textsubscript{L} fused with MBP through the D\textsubscript{K}–MBP cleavage site and expressed this in \textit{E. coli} cells expressing chaperone proteins (Fig. 1A). A previous report demonstrated expression of soluble and active MBP-tagged hEK\textsubscript{L} [26]. However, we found that MBP-hEK\textsubscript{L} was unable to self-cleave, indicating an absence of the enzymatic activity (Figs. 1A and 1B). To test whether removal of MBP could restore the hEK\textsubscript{L} activity, an hEK\textsubscript{L} variant was constructed by replacing the D\textsubscript{K} with the TEV protease recognition site (ENLYFQ). However, hEK\textsubscript{L} obtained by TEV cleavage of MBP-hEK\textsubscript{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\textsubscript{L} indicated that the refolded enzyme was partially active (Fig. S2).

\textbf{Abbreviations:} D\textsubscript{K}, Aspartic Acid\textsubscript{4} Lysine; EK, enterokinase; bEKL, bovine enterokinase light chain; hEK\textsubscript{L}, human enterokinase light chain; IPTG, isopropyl β-D-1–thiogalactopyranoside; MBP, maltose-binding protein; TEV, tobacco etch virus.

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These results demonstrated that MBP fusion enhances the solubility of hEKl but does not allow its correct folding. We speculated that hEKl 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 hEKl, 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 hEKl was successfully detected, although at a low level (7.9 % of total MBP-DaK-hEKl), in cells grown at 20 °C (Fig. 1c). Use of the C112S mutated hEKl dramatically improved the ratio of self-cleaved hEKl 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 hEKl 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, hEKl was not visible in the SDS-PAGE gel even when hEKl activity was observed. However, as shown in Fig. S3, when inactivated hEKl was produced by TEVp, hEKl was visible in the SDS-PAGE gel. Therefore, we assumed that the visibility of hEKl in the SDS-PAGE gel was influenced by its folding.

We further monitored the time profiles for cell growth and enzymatic activity of hEKl, C112S (Fig. 2a and b). After 27.5 h of culture, the cell growth reached the maximum (2.87 OD600) and then sharply decreased. At that time, the hEKl activity in the soluble fraction reached the maximum value (372 U/mL) and then decreased to ~22 U/mL. In contrast, hEKl 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 hEKl C112S from culture supernatants. The culture supernatant of E. coli SHuffle expressing pET-30a-MBP-DaK-hEKl, C112S and pACYC-GroEL/ES was loaded on the affinity chromatography (HisTrap™) 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 hEKl (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 hEKl at high purity (>99 %) and yield (>99 %) using a simplified one-step method. Purified hEKl, C112S had affinity to DaK-ηa with Kd = 0.287 ± 0.079 mM, turnover number Kcat = 6.725 × 104 ± 1.230 × 104 s⁻¹, and catalytic efficiency Kcat/Kd = 2.385 × 105 M⁻¹ s⁻¹.

In conclusion, we could purify soluble and active hEKl 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 hEKl from E. coli.

**Author contributions**

Y.S.K, H.-J. 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
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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