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# Analysis and Prevention of Inclusion Body in *E. coli* Over-expression Systems Using Short Peptide Tags

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## Research aims

*Escherichia coli* (*E. coli*) can yield high amount of proteins at a low cost, and it is thus frequently used for the expression of recombinant proteins. However, many recombinant proteins express as inclusion bodies, which are insoluble protein aggregates forming in the interior of *E. coli* cells. Protein recovered from inclusion bodies can sometimes be refolded into their native states, but, due to their diverse physicochemical properties, a versatile refolding method is still lacking. Here, we report a systematic analysis of the relationship between *in vivo* inclusion body formation and *in vitro* solubility using SEP-tags, which are short Solubility Enhancing Peptide tags consisting of a glycine and charged amino acids, and which were developed in our laboratory (ref 1, 2; patent pending). In addition, we examine the ability of SEP-tags to prevent inclusion body formation in *E. coli*.

## Methods

We designed three SEP-tags with different lengths (containing 3, 6, and 9 charged amino acid residues), and fused them to the C-terminus of 6 model proteins: CAD 1-86 C34S, GLuc (ref 3), N-intein (ref 4), GFP (ref 4), VanX E181A, and P57. SEP-tags containing arginines were fused to the basic protein (CAD 1-86 C34S); whereas SEP-tags containing aspartic acids were fused to the acidic proteins (N-intein, GFP, VanX E181A, GLuc, and P57) (Table 1). All of the recombinant variants were overexpressed in *E. coli* strains BL21 (DE3) pLysS, and JM109 (DE3) pLysS at 37, 30, and 25°C. The effect of SEP-tags on inclusion body formation was analyzed by SDS-PAGE and gel band densitometry. Furthermore, we measured the effect of SEP-tags on the activities of GFP, VanX, and GLuc.

## Results and Discussions

SEP-tags are essential in this study, which was aimed at rationally analyzing the effects of *in vitro* protein solubility on inclusion body formation, because they can modulate *in vitro* protein solubility without (much) affecting their folding structure or stability. Overall, the SEP-tags significantly reduced the formation of inclusion body. One example is shown in Figure 1 where SEP-tags containing 3, 6, and 9 aspartic acids were attached to N-intein, an acidic protein with a molecular weight of 14.4 kDalton. The extent of reduction was directly dependent on the number and type of charged amino acids contained in the SEP-tags (Figs. 1 and 2). These observations confirm that the formation and/or prevention of inclusion body is intimately related to protein solubility, and that improvement in solubility can prevent or significantly reduce the formation of inclusion body. We observed very similar effect in both JM109 (DE3) pLysS and BL21 (DE3) pLysS strains of *E. coli.*

The types of the charged residues included in the SEP-tags strongly influenced its effect on preventing inclusion body. SEP-tags containing aspartic acid increased the expression of GLuc in the soluble fraction to 80%, however, the increase resulting from arginine tags were merely 13%. This result suggests that the compatibility of the physicochemical nature of the target protein with the SEP-tag

**Table 1.** List of model proteins.

| | MW(kDa) | pI | NoTag | C3D | C6D | C9D | C3R | C6R | C9R |
|---|---|---|---|---|---|---|---|---|---|
| CAD 1-86 C34s | 10.0 | 9.8 | ○ | — | — | — | ○ | ○ | ○ |
| N-intein | 14.4 | 4.4 | ○ | ○ | ○ | ○ | — | — | — |
| GLuc | 18.6 | 6.7 | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| VanX E181A | 23.6 | 5.8 | ○ | ○ | ○ | ○ | — | — | — |
| VanX | 23.6 | 5.6 | ○ | — | — | ○ | — | — | — |
| GFP | 27.2 | 5.8 | ○ | ○ | ○ | ○ | — | — | — |
| P57 | 56.7 | 5.2 | ○ | ○ | ○ | ○ | — | — | — |

Variants that were constructed are indicated by a circle (○). All of the variants were expressed using a pAED4 expression vectors (ref 5～7). MW (kDa) and pI indicate, respectively, the molecular weight and the isoelectric point calculated from the untagged (original) sequences. VanX E181A is an inactive mutant of VanX, which was used because the wild type VanX exhibits a lysis activity when expressed in *E coli*. The gene encoding the model proteins were introduced using an NdeI and an NcoI restriction site. The SEP-tag was located downstream the DNA sequence encoding the model protein.

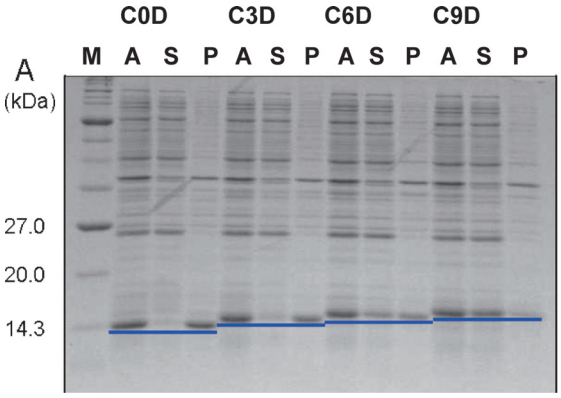


**Fig. 1.** SDS-PAGE of N-intein expressed in BL21(pLysS) at 37°C. M, A, P, and S indicate the molecular weight marker, the whole cell, the precipitate (inclusion body) and the supernatant, respectively. C0D is the untagged protein. C3D, C6D and C9D indicate, respectively, N-intein N tagged with 3, 6, and 9 aspartic acid residues. The blue line indicates the N-intein band.

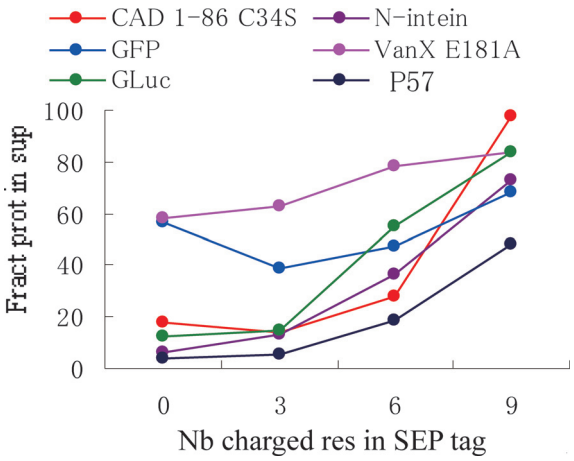


**Fig. 2.** Fraction of protein expressed in the supernatant.

residues (either acidic or basic) is essential in enhancing protein solubility and thereby reducing the amount of protein expressed as inclusion body. As a practical rule, SEP-tags containing arginine should be used for basic proteins and aspartic acid for acidic proteins.

The expression temperatures are well known to influence the formation of inclusion body, and we indeed observed some influence on the effect of the SEP-tags. For example, although, at 37°C, the addition of SEP-tags had essentially no effect on the fraction of CAD 1-84 C34S and P57 expressing as inclusion body; at 25°C the fractions of CAD 1-84 C34S, and P57 in the soluble fraction increased to, respectively, 80% and 44% upon addition of the SEP-tags. We hypothesize that SEP-tags had no effect at 37°C, because both proteins were unfolded or partly unfolded, which decreased the protein's solubility, whereas SEP-tags were efficient at 25°C where the proteins are stably folded.

**Table 2.** Relative activity of proteins tagged with SEP-tags relative to that of non tagged proteins.

| SEP-tagged proteins | Relative activity (%) |
|---|---|
| GFP C9D | 68 |
| VanX C9D | 79 |
| GLuc C9D | 130 |

Finally, the activity of SEP-tagged GFP, and VanX dropped by 32% and 21%, respectively, whereas that of SEP-tagged GLuc increased by 30% (Table 2). We considered that the discrepancies were within the limit of our experimental settings and that the tags did not significantly influence the function of these proteins.

## Conclusions

Here, we have used SEP-tags to show, in a quantitative manner, that protein solubility is an essential factor determining the formation of inclusion body in *E. coli*. SEP-tags, which can regulate *in vitro* protein solubility, appears to be a versatile method for reducing the formation of inclusion body, leading to increased yield of soluble, functional native proteins. As for SEP-tag design asparagines should be used for acidic proteins and arginines for basic proteins (ref 2, patent pending).

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