

Preparing of Active Recombinant AEP free of Acid-activation.

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Method Article

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Abstract

Asparaginyl endopeptidase is a useful biocatalyst for site-specific protein bioconjugation. However, existing recombinant protocol is lengthy requiring cap removal of the zymogenic enzymes through acid activation and extra chromatographic steps. Here, we describe an activation-free approach for AEP preparation where the cap and core domains are prepared as separate entities during gene expression. An AEP variant from *Oldenlandia affinis* (OaAEP1b-C247A) can be prepared without the need of acid treatment giving a yield of ~1.5-2.2 mg of enzyme per litre of culture. Likely because of a decrease in chromatography time and an increase of homogeneity, activity of the split AEP was found to be ~3-fold higher than those obtained using existing protocols, highlighting its potential usefulness.

Introduction

Asparaginyl endopeptidases (AEPs) are ideal catalysts for protein bioconjugation because they have a short recognition sequence and catalytic efficiency (1, 2). Contemporary research where AEP is applicable includes the study of post-translation modifications in cell biology research, protein-drug conjugation for the production of biopharmaceuticals, peptide-ligation for the semi-synthesis of proteins, bioconjugation to solid supports for the immobilisation of proteins (3, 4, 5). However, application of AEPs has been limited by their complex preparation protocol. The genes of recombinant AEPs are typically expressed in fusion with its cap domain which silences enzyme activity by blocking access to the active site (1, 6, 7). The cap domain, therefore, needs to be removed by a low pH treatment, which takes up to 2 hours and requires multiple chromatographic steps for purification (up to 4 steps) (1, 7). Here, we outline a new activation-free protocol for recombinant AEP preparation that prepares the core enzyme and cap domain as split entities (**Figure 1**). This simplified procedure enables core AEP to be prepared in no more than two chromatographic steps whilst offering excellent catalytic efficiency.

Reagents

Deionised water (dH_2O) was obtained from an Elga[®] PURELAB Chorus 2 system.

TCEP-HCl, kanamycin sulfate, imidazole were purchased from Fluorochem.

Ethylenediamine tetraacetic acid, disodium salt dihydrate (EDTA), sodium phosphate dibasic and sodium chloride were purchased from ThermoFisher Scientific.

Sodium acetate was purchased from Melford.

Peptide substrates were purchased from ISCA Biochemicals or GenScript or synthesised *in situ*.

Calcium chloride and isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Melford[®] 30% acrylamide/bis-acrylamide solution and SYBR safe was purchased from Merck Sigma-Aldrich.

Buffers, nucleotide triphosphate and PrimeSTAR Max DNA polymerase were purchased from Takara.

All restriction enzymes were purchased from ThermoFisher Scientific. NEBuilder® HiFi DNA assembly master mix was purchased from New England Biolabs.

The gene optimized for expression in *E. coli*, encoding a protein composed of a N-terminal His₆ tag, 76-residue human ubiquitin and OaAEP1-C247A (from residues 24-474) was inserted into the coding region (Ncol-Ndel) of the pET-28b (+) vector (Genscript).

Equipment

Shaking incubator for bacterial growth

FPLC system including Ni sepharose and Superdex®200 Prep Grade

Procedure

Preparation of the plasmid

1. Amplify the genes of the hexahistidine-ubiquitin (His₆-Ub) tagged core OaAEP1-C247A and the cap domain by use of the following primers:

His₆-Ub tagged core OaAEP1-C247A

P1: AAGCTTGC GGCCGC ACTCGAGGAGATCCGGCTGCTAACAAAGCC

P2: TCTCCTTCTTAAAGTTAACAAAATTATTCGGATCCTAGTCGTTGCCGGTTGC

cap domain

P3: TTTGTTAACTTAACAGAAGGAGATCATATGAACATACCAGCCTGGATGGTAACGC

P4: CTCGAGTGC GGCCGCAAGCTTTACGGGATGCTCGCGCACG

2. Insert the PCR products into a pET28b vector by use of the Gibson Assembly and transform into DH5α *E. coli*. Confirm the DNA sequences by Sanger sequencing (e.g. through Eurofins Genomics).

3. Introduce the mutation D29E by use of the following primers:

P5: CGCGAAGAAAGCGTGGGTACCCGTTG

P6: GTGCAGATATTCAACCGGTACGGCGGCCACC

4. Treat the PCR product with DpnI digest enzyme. Extracted plasmid from DNA agarose gel. Transformed the plasmid into DH5 α *E. coli*. Confirm the DNA sequences by Sanger sequencing (e.g. through Eurofins Genomics).

Gene Expression

5. Introduce the plasmid to Shuffle® T7 *E. coli* by heat-shock transformation.

6. Select a single colony from the agar plate to inoculate 10 mL of LB medium that is added with 50 μ g/mL kanamycin and then incubate at 30 °C overnight (16-20h) with shaking at 220 rpm.

7. Inoculate the starter culture into a LB media that contains 50 μ g/mL of kanamycin at a ratio of 1:100 (10 mL into 1 L) at 30 °C until an OD₆₀₀ value of 0.6-0.7 is reached.

8. Induce gene expression by adding IPTG (0.4 mM) at 16 °C for 18 h and isolate the cell pellet by centrifugation (500 rpm/min, 15 min, 4 °C).

AEP preparation

9. Resuspend the cell pellet from a 1.0 L culture in 20 mL of lysis buffer (50 mM sodium phosphate, 300 mM NaCl, pH 8.0) with lysozyme (0.1 mg/mL), PMSF (35 μ g/mL), DNase I (5 μ g/mL)

10. Sonicate on ice (Amplitude: 39 %, 5 min, 5 s pulse ON, 15 s OFF) and subject the lysate to centrifugation (27,000g, 4 °C, 15 min).

11. Introduce the supernatant to 2 mL of Ni-NTA resin (Bio-Rad) in a gravity flow column (Bio-Rad).

12. Wash the column three times with lysis buffer that contains 10 mM imidazole (15 mL each).

13. Elute the remaining bound protein with 15 mL of lysis buffer that contains 300 mM imidazole.

14. Concentrate the eluted protein using a VIVASPIN centrifugal filter (10 kDa MWCO) (Cytiva).

15. Purify by size exclusion chromatography (Superdex 75, GE Healthcare) that had been pre-equilibrated in 50 mM sodium acetate buffer (pH 4.0), 150 mM NaCl, 0.5 mM TECP and 1 mM EDTA.

16. Combine fractions of desired protein and concentrate using a VIVASPIN centrifugal filter (10 kDa MWCO) (Cytiva).

17. Store at -80 °C with 5% (v/v) glycerol.

18. Perform SDS-PAGE analysis and estimate enzyme concentration by nanodrop UV-Vis A₂₈₀ (**Figure 2**).

Kinetic characterisations

19. Check enzyme activity by performing peptide ligation assays in 100 µL reaction mixtures containing 50 mM Na₂HPO₄ (pH 7.0, 5 mM EDTA), supplemented with 1 mM peptide GLP, the AEP (10 or 40 nM) and peptide substrate (20 to 240 µM).

20. Perform each reaction in duplicate at 25 °C by use of the microplate reader (FLUOstar Omega).

21. Calculate reaction velocities by converting the slopes of fluorescence intensity change at 450 nm, which corresponds to the concentration increase of the ligated peptide product.

22. Fit data to equation 3 using GraphPad Prism 9.0.0 (GraphPad) to estimate the kinetic parameters (k_{cat} and K_M , **Figure 3**).

$$v = V_{max} [S] / (K_M + [S]) \quad (3)$$

Troubleshooting

STEP: Gene expression

PROBLEM: No expression observed on SDS-PAGE

POSSIBLE REASON: Temperature for gene expression is too high/low; No/wrong antibiotic

SOLUTION: Bring to 30 °C; Check and use the correct antibiotic

PROBLEM: No colonies on the agar plate

POSSIBLE REASON: No/wrong antibiotic

SOLUTION: Check and use the correct antibiotic

PROBLEM: No colonies on the agar plate

POSSIBLE REASON: Pipette the plasmid up and down into the competent cell for mix

SOLUTION: Gently mix by flicking the tube bottom by finger

PROBLEM: No colonies on the agar plate

POSSIBLE REASON: Excessive plasmid added leading to the low transformation efficiency

SOLUTION: Diluted the stock plasmid to around 10 ng/ μ L and add 1 μ L to the competent cell

STEP: Ni-NTA column

PROBLEM: Target protein stays in the pellet

POSSIBLE REASON: Incomplete cell lysis

SOLUTION: Set the off to 15 s and keep the cell lysate chilled during sonification; Ensure temperature remains at 16 °C after IPTG addition

PROBLEM: Proteins are not eluted

POSSIBLE REASON: Insufficient imidazole in elution buffer

SOLUTION: Bring imidazole concentration to 300 mM

STEP: Size exclusion chromatography

PROBLEM: SDS-PAGE illustrates two bands

POSSIBLE REASON: Two cut sites identified.

SOLUTION: Remove potential cut sites of AEP by performing site-directed mutagenesis described in “Preparation of the plasmid”

PROBLEM: Cap domain not isolated

POSSIBLE REASON: The buffer pH is too high

SOLUTION: Bring to pH 4.0

PROBLEM: Low protein yield from size exclusion chromatography

POSSIBLE REASON: Protein solution is over-concentrated after Ni-NTA, resulting in precipitation

SOLUTION: Keep protein concentration below 0.2 mg/mL

Time Taken

The whole procedure will take about 6-7 days: 2 days for gene cloning, 1 day for transformation, 2 days for gene expression, 1 day for protein purification.

Anticipated Results

Highly active OaAEP1 variant is anticipated.

References

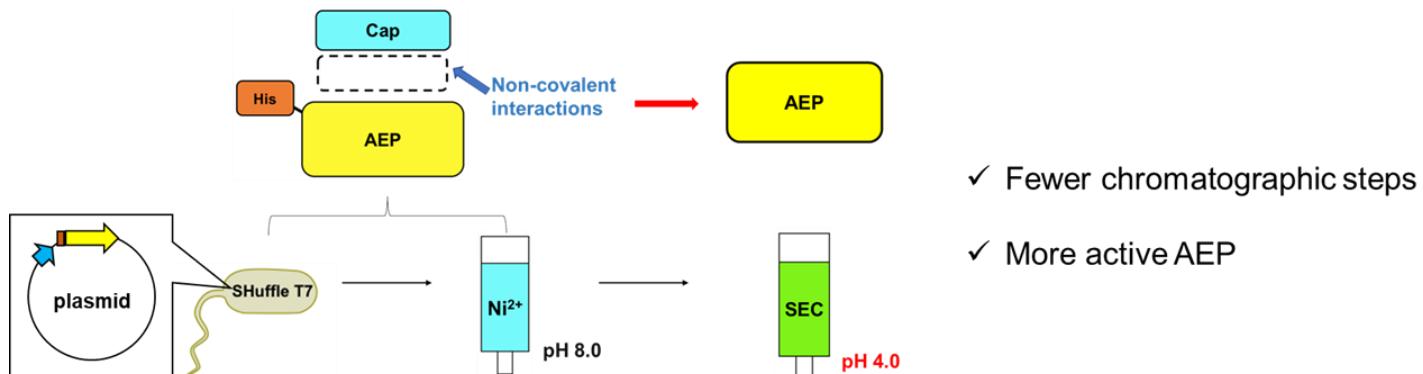
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Figures

(A) This Work



(B) Previous Work

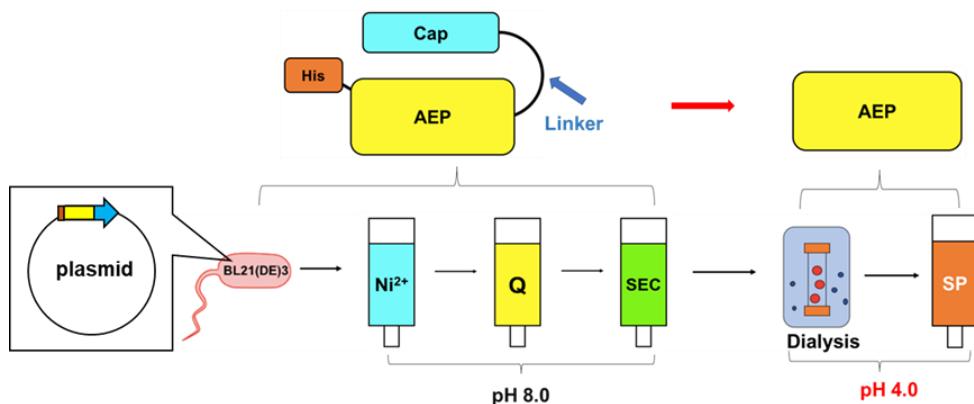
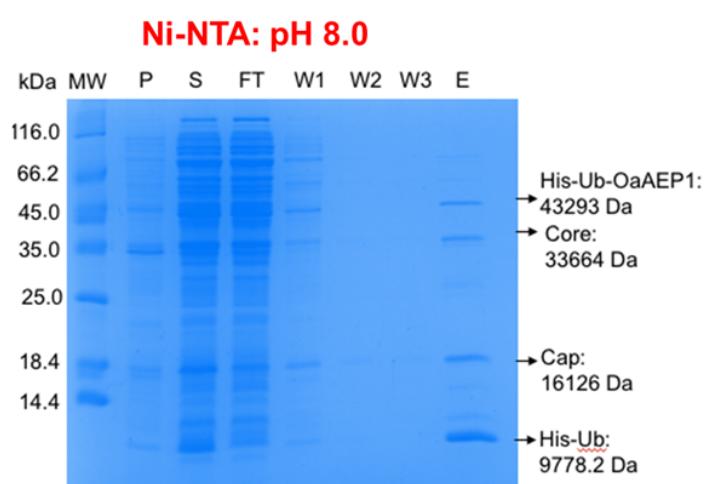


Figure 1. (A) Scheme of core AEP preparation without acid activation. The core AEP isolated by this approach requires only 2 chromatographic steps, instead of 4 as in previous approach **(B)** (see Ref 1, 6, 7).

Figure 1

(A) Scheme of core AEP preparation without acid activation. The core AEP isolated by this approach requires only 2 chromatographic steps, instead of 4 as in previous approach **(B)** (see Ref 1, 6, 7).

(A) SDS-PAGE analysis



(B) MS analysis

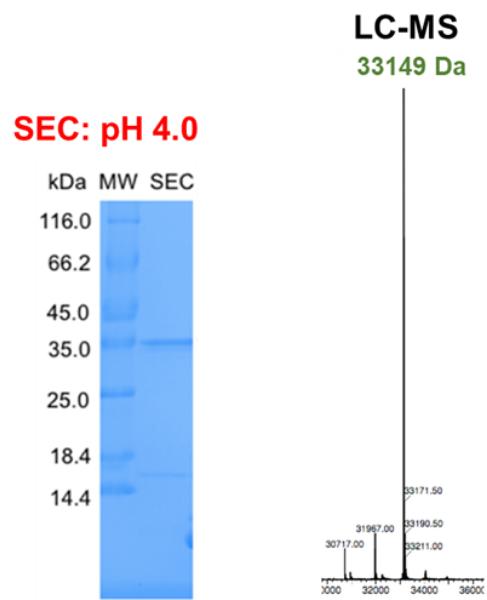


Figure 2. (A) SDS-PAGE analysis of the split AEP isolated Ni-NTA (pH 8.0) and size exclusion (pH 4.0) chromatography, and (B) LC-MS of the core AEP.

Figure 2

(A) SDS-PAGE analysis of the split AEP isolated Ni-NTA (pH 8.0) and size exclusion (pH 4.0) chromatography, and (B) LC-MS of the core AEP.

Split-OaAEP1-D29E, C247A nucleotides sequence

ATGGGCATGGCGCACCAACCACCATGCAGATCTCGTAAACCCCTGACCGGCAAGACCATTACCCCTGGAAGT
 GGAACCGAGCGACACCATCGAGAACGTGAAAGCGAAGATCCAAGACAAAGAAGGTATTCCGCCGGATCAGCAACGTCTG
 ATTTTGCGGGCAAGCAGCTGGAGGACGGTCGACCGTGAAGCGATTACAACATCCAAAAGAAAGCACCCTGCATCTGGT
 GCTGCGTCTCGTGGCGCGTGAACGGTGAATTATCTGCACCTGCCGAGCGAGGTGTCGTTCTTCGTCCGCAGGA
 GACCAACGACGATCACGGCGAAGAAAGCGTGGGTACCCGTTGGCGGTTCTGATTGCGGTAGCAAGGGCTACGCGAAC
 TATCGTCATCAGGCGGGCGTGTGCCATCGTACCAAAATCTGAAACGTGGTGGCCTGAAGGATGAGAACATCGTGGTTTC
 ATGTACGACGATATTGCGTATAACGAAAGCAACCCCGTCCGGGTGTTATCATTAACAGCCCGCACGGCAGCGATGTGATG
 CGGGTGTCCGAAAGATTATACCGCGAGGAAGTGAACCGCGAAGAACCTCCTGGCGGCGATCTGGTAACAAAAGCGC
 GATTACCGGTGGCAGCGCAAGGTGGTGACAGCGTCCGAACGATCACATCTTATTACTATACCGACCACGGTGCACG
 GGGCGTTATCGGTATGCCGAGCAAGCGTACCTGTATGCCGACGAGCTGAACGATGCCGTGAAGAAAAAGCACCGCAGCG
 GTACACTACAAAAGCCTGGTGTCTATCTGGAGGCCTGCAGAACGCGCAGCATGTTGAGGTATCCTGCCGGAAAGATCTGA
 ACATTTACCGCCTGACCAGCACCAACACCACCGAAAGCAGCTGGCCTACTATTGCCCGCGCAGGAGAACCCGCCCG
 CGGAATATAACGTGTGCCCTGGCGACCTGTTAGCGTTGGCTGGAGGACAGCGATGTGCAGAACAGCTGGTACGA
 AACCCCTGAACCAGCAATATCACCACGTTGATAACGTATTAGCCACCGCAGCCACCGCACCGTAACCGTAACCTGAAACT
 GGGCGAGGAAGGTCTGTTGTACATGGCAGCAACCCCGCGAACGACTAAGGATCCAAATAATTGTTAACCTTAA
 GAAGGAGATATCATATGAACTATACCGCCTGGATGGTAACCGCCTGACCCCGAGCAGCATCGTGGTTAACAGCGTACG
 CGGATCTGCTGCACCTGTGGAGAAATTCCGTAAGGCACCGCGTAAAGAGGAAGCGCAGACCCAAAT
 TTGTTAAGGCATGAGCCACCGTGTTCACATCGACAGCAGCATCAAACGTATTGGCAAGCTGCTGTTGGTATCGAGAAATG
 CACCGAAAITCTGAACCGGTTCGTCCGGCGGGTCAACCGCTGGTTGACGATTGGCGTGCCTCGTAGCCTGGTGGTA
 CCTTGAGACCCACTCGCGCAGCCTGAGCGAACACGGTATCGTCACACCCGTACCATCGAACATTGCAACCGGGTA
 TTAGCGAGGAACAGATGGCGGAAGCGCGAGCCAAGCGTGCAGCATCCCGTAA

Split-OaAEP1-D29E, C247A amino acids sequence:



(Arrow indicates the cleavage site of truncated active AEP)

Figure 3

Gene and Protein sequences