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Enhancement of Recombinant Human ADAM15 Disintegrin Domain Expression Level by Releasing the Rare Codons and Amino Acids Restriction

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Abstract This study was aimed at increasing the production of the recombinant human ADAM15 disintegrin domain (RADD) in *Escherichia coli* by releasing the rare codons and restricting amino acid residues. Three different strategies for increasing RADD production were examined: to select the suitable host strain, to optimize the rare codons, and to delete the amino acids residues. The total fusion protein glutathione-S-transferase (GST)-RADD concentration of 298 mg/l and 326 mg/l were achieved by selecting *E. coli* Rosetta (DE3) as the host strain and by changing GGA to GGC at the GST-RADD cassette, respectively. The RADD concentration was increased by 35.7% by eliminating the "Pro-Glu-Phe" residues at the GST–RADD junction. By combinational utilizing the preferred codon introduction and amino acid sequence optimization in *E. coli* Rosetta (DE3), the highest RADD concentration of 68 mg/l was achieved. The proposed strategy may provide an alternative approach for other enhanced recombinant protein production by *E. coli*.

Keywords Recombinant ADAM15 disintegrin domain (RADD) · Rare codon · Amino acids residues

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Introduction

*A D*isintegrin *A*nd *M*etalloproteases (ADAM) proteins are a family of multifunctional proteins containing disintegrin and metalloproteinase domains that perform both adhesive and proteolytic functions in cell–cell and cell–matrix interactions [1]. ADAM15 is unique among these proteins in having an Arg-Gly-Asp (RGD) motif in its disintegrin-like domain. It was found that ADAM15 could interact with integrins αvβ3, α5β1, and α9β1 to enhance cell adhesion [2–4]. In addition, the recombinant ADAM15 disintegrin domain exhibited high inhibitory activities on angiogenesis, tumor growth, metastasis, and pathological neovascularization [5, 6]. All these results suggested that ADAM15 protein exhibited a potential for diagnostics of human cancers [7], and recombinant ADAM15 disintegrin domain might be a drug lead compound. To study the pharmacology and anticancer mechanism of recombinant ADAM15 disintegrin domain, it is necessary to acquire enough bioactive ADAM15 protein by developing a highly efficient expression system. In the past decades, ADAM15 or its disintegrin domain has been successfully expressed in *Escherichia coli*, *Pichia pastoris*, *Chinese Hamster Ovary (CHO) cells* and other histiocytes [6, 8–11]. However, high expression level of bioactive ADAM15 (milligram per liter class) by *P. pastoris* or *E. coli* DH5α could not be achieved.

The production of functional recombinant protein in host strain was affected by numerous factors. These factors could be categorized as three: (1) the nutritional and environmental conditions [12], (2) the gene of aim protein, and (3) the host strain. To enhance the recombinant proteins production, the following two major operational strategies were generally used singly or in combinational. The first strategy was to optimize those environmental factors such as growth temperature, medium composition, and concentration of gene expression inducers. The second strategy was mostly involved with the genetic engineering of the target protein, and actually the characteristics and relationship of the target protein and its host cell, are also the key factors for the enhanced production of recombinant protein [13]. For example, the excessive rare codon usage in the target gene has been implicated as a cause for low-level expression as well as truncation products [14, 15]. The effect seems to be more severe when the multiple rare codons occurred near the amino terminus. During the translational process, the preferred codons in highly biased genes optimally bind to the most abundant iso-accepting transfer RNA (tRNA) in host strains, thus increasing translational efficiency by improving the speed and accuracy of protein synthesis [16]. As comparison, the insufficient preferred codons could lead to the recombinant protein gene translational stalling, termination, frame-shifting, or amino acid mis-incorporation.

On the other hand, it has been shown that fusion proteins such as glutathione-S-transferase (GST) fusion proteins have the advantage of providing a more favorable gene constructing organization for high expression of soluble proteins. The concentration of the target protein was affected by thrombin cleavage rate. For example, a strategy by improving the thrombin cleavage rate for high-level expression of human interferon α (IFNα) as the GST fusion protein in *E. coli* was referred [17]. The recombinant bovine lactoferricin (LfcinB) was expressed as the GST fusion protein, which contains a Factor Xa cleavage site, and a thrombin cleavage site, but functional LfcinB was only cleaved by thrombin protease, not by Factor Xa [18].

This study focused on increasing production of the recombinant ADAM15 disintegrin domain (RADD) in *E. coli.* To achieve this target, three strategies were examined, namely: (1) choosing *E. coli* Rosetta (DE3) as the host strain based on the coding sequence analysis of RADD protein; (2) releasing the rare codons restriction in the fusion protein GST-RADD cassette; (3) improving the cleavage efficiency of thrombin. The results showed that the highest production of RADD could be accomplished by combinational utilization of these strategies.

Materials and Methods

Chemicals and Strains

Ampicillin and chloramphenicol were purchased from Sigma-Aldrich (Steinheim, Germany). The restriction polymerases *Bam*HI and *Not*I, T4 DNA ligase, and the Site-Directed Mutagenesis Kit were purchased from Takara Co. (Japan). Anti-human ADAM15 ectodomain antibody and Donkey anti-goat IgG were purchased from R&D Systems Co. (USA) and Santa Cruz Biotechnology (USA), respectively. Glutathione-Sepharose 4B and Sephadex G75 (2.6×60 cm) were from Amersham Pharmacia Biotech (Sweden). All inorganic compounds were reagent grade or higher quality.

The plasmid pGEX-4T-1 was kindly presented by Prof. Huazhong Li (Jiangnan University). *E. coli* XL-1-Blue, BL21 (DE3), and Rosetta (DE3) were purchased from Invitrogen.

Construction of Recombinant Plasmid pGEX-ADAM15

A 273-bp cDNA fragment that encodes the entire disintegrin domain of ADAM15 (Met^{420}-Glu^{510}) [19] was a gift from Dr. Qin Chen (Shanghai Institute of Materia Medica) and used as the template for polymerase chain reaction (PCR). The primer was 5′-GGCGTCGACTCATGGCTGCTTTCTGCGGAAATATG -3′ (P1), which contains a *Sal*I site (underlined), and 5′- ATAGCGGCCGCTCACTCGCCATCCCCTAGGCTGAC -3′ (P2), which contains a *Not*I site (underlined). The resulting PCR products were digested with *Sal*I and *Not*I and then ligated to the corresponding sites of the vector pGEX-4T-1 by T4 DNA ligase. The ligation product was then transformed into the competent cells of *E. coli* XL-1-blue, and the recombinant colonies were taken for overnight culture. The recombinant expression plasmid pGEX-ADAM15 was extracted and verified by restriction enzymes *Sal*I and *Not*I and then transformed into *E. coli* BL21 and Rosetta (DE3).

Construction of Engineered Plasmid pGEX-Δ-ADAM15

Following the manual of the Site-Directed Mutagenesis Kit, the pGEX-ADAM15 expression vector was used as the DNA template for PCR-SDM to mutate the codon GGA to GGC by using a pair of mutagenic primers:, P3:5-′ATGGCTGCTTTCTGCGG CAATATGTTTGTC -3′, P4:5-′ACCCGGGAATCCACGCGGATCCGATTT -3′, and then the plasmid pGEX-Δ1-ADAM15 was obtained. The plasmid pGEX-Δ2-ADAM15 was constructed in the same way but using the different mutagenic primers: P5: 5-′ ATGGCTGCTTTCTGCGGAAAT-3′,P6: 5-GGA GCC ACG CGG AAC CAG-3′. Finally, the engineered plasmid pGEX-Δ-ADAM15 was amplified by the PCR reaction, with the DNA template and the mutagenic primers being pGEX-Δ2-ADAM15 and P5 and P6, respectively. The engineered plasmids pGEX-Δ1-ADAM15, pGEX-Δ2-ADAM15, and pGEX-Δ-ADAM15 were verified by DNA sequencing analysis.

Expression of GST-RADD Protein in Recombinant Rosetta (DE3)

A single colony of transformants was grown with 50-ml Luria-Bertani (LB) medium with 50 μg/ml chloramphenicol and 100 μg/ml ampicillin in a 250-ml flask. The culture was grown at 37 °C in an incubator shaker at 250 rpm for about 3 h until the culture reached at OD_{600}=0.8–1.0. To induce expression, the culture was supplemented with 0.05 mM isopropyl-β-D-thiogalactoside (IPTG) and incubated for 6 h at 30°C. The culture was

harvested by centrifugation, washed, and resuspended with ice-cold phosphate-buffered saline (PBS). The pellet was mixed with PBS buffer at a ratio of 1:5 and boiled for 5 min. The level of protein expression was assayed on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The optimal expression conditions, such as IPTG concentration and addition time, retaining time, and induction temperature, were determined in the shaking flasks.

Purification of GST-RADD Protein

To purify the GST-RADD protein, the cultures were harvested and centrifuged at 10,000×*g* for 10 min at 4 °C, then the pellet was resuspended in 0.4-l ice-cold PBS and lysed by gentle sonication (100 W, 3 min, ACX 400 sonicator, 20 kHz, Sonic and Materials, Newton, MA, USA) under ice-water bath. The cell debris was removed by centrifugation at 15,000×*g* for 20 min at 4 °C and then the supernatant was collected. The supernatant containing fusion protein was filtered through a 0.45 μm Millipore membrane and the filtrate was loaded onto a glutathione Sepharose 4B column (Amersham-Pharmacia) preequilibrated with Tris–HCl buffer (100 mM Tris–HCl, pH 8.3, 10 mM NaCl, 0.5 mM DTT). Nonspecifically bound proteins were removed by washing with buffer, and the bound fusion proteins with GST tag were eluted with 100 mM Tris–HCl (pH 8.3) buffer containing 10 mM reduced glutathione. The elution of the fusion protein was repeated three times and the eluates were pooled together. The concentration of eluted GST-RADD was calculated by the BCA (bicinchoninic acid) assay.

Cleavage of GST-RADD Fusion Protein

The recombinant protein GST-RADD was digested by thrombin protease (10 cleavage units per milligram fusion protein) in elution buffer (100 mM Tris–HCl, pH 8.3, 10 mM NaCl, 1 mM EDTA, and 1 mM DTT) for 8 h at 4 °C. The reaction mixture then passed through a Sephadex G-75 gel filtration chromatography with PBS (pH 7.4) at 2.5 ml/min. The purified RADD was collected and then loaded on the 16.5% Tricine-SDS-PAGE. The protein was monitored by SDS-PAGE throughout the purification procedure.

Western Blot Analysis, Amino Acid Analysis, and Mass Spectroscopy (MALDI TOF)

Western blotting analysis was performed as previously described [20]. To evaluate the proper processing of RADD, N-terminal sequencing of the protein was conducted by Edman degradation on an Applied Biosystems ABI Procise 492cLC sequencer using standard techniques. RADD protein was further analyzed for molecular mass in Shanghai Applied Protein Technology (Shanghai, China).

Biological Assay

The biological activity of the RADD was evaluated by its ability to inhibit the proliferation and migration of human microvascular endothelial cells (HMEC-1) (Inserm U553, Hospital Saint-Louis, Paris, France). The proliferation was determined by the MTT assay in 96-well plates [21], and the inhibition ratio was calculated as $[1 - (A_{570\ \text{treated}}/A_{570\ \text{control}})] \times 100\%$. The migration assay was performed in 24-well culture plates according to the method described by Trochon-Joseph [5].

Results

Enhancement of GST-RADD Expression Level with *E. coli* Rosetta (DE3) as Host Strain

As shown in Fig. 1A, the expression plasmid pGEX-ADAM15 was transformed into *E. coli* BL21 (DE3) and then cultured in LB medium, a band of 37-kDa GST-RADD (lane 3) and 26-kDa free GST protein (lane 2) were obtained after inducing by IPTG for 6 h at 37 °C. About 119 mg/l fusion protein (lane1) was achieved by using a glutathione Sepharose 4B column and determined by BCA assay. However, a relatively low RADD protein expression level and a bioactivity on HMEC-1 cells were observed.

To enhance the expression level and bioactivity of RADD, a detailed comparative analysis of the coding sequence of ADAM15 disintegrin domain was performed using the "*E. coli* Codon Usage Analyzer 2.0" software (http://www.lifesci.ucsb.edu/~maduro/codonusage/usage2.0c.htm). It was found that the rare codons GGA (Gly6, Gly46, and Gly76), GGG (Gly66 and Gly88), CCC (Pro27, Pro47 and Pro82), TTG (Leu32 and Leu70), CTA (Leu87), AGG (Arg37), and AGA (Arg65) in the coding sequence of ADAM15 disintegrin domain (Fig. 1B). A complete compilation of codon usage of the sequences placed in the GenBank database can be found at website http://www.kazusa.or.jp/codon/, and *E. coli* displays frequencies of about 0.6% of rare codon. To achieve a high-level expression of the soluble RADD, a rare codon optimizer strain, *E. coli* Rosetta (DE3), the derivative of BL21 (DE3) containing a plasmid of pRARE (Cm^R) encoding rare codon tRNAs, including these six different codons, was introduced as the host strain.

The optimal GST-RADD production conditions were as follows: 0.1 mM IPTG was supplemented to the *E. coli* Rosetta (DE3) culture broth when the cell concentration reaching at OD_{600} 0.8 under 37 °C, then the temperature was shifted to 30 °C and maintained for 6 h. About 298 mg/l GST-RADD protein from *E. coli* Rosetta (DE3) (66% of total protein) and 42 mg/l RADD protein were obtained, and these values were 150.4% and 133.0% higher than those when using *E. coli* BL21 (DE3), respectively.

Confirmation of RADD Protein

A series of experiments were performed to confirm the correct expression in strain *E. coli* Rosetta (DE3): (1) purified RADD was analyzed by high-performance liquid chromatography with SephadexG-75; a single peak corresponding to RADD was observed (Fig. 2A) and the purity more than 95% (Fig. 1A); (2) the purified RADD could be immuno-reactive with antihuman ADAM15 antibody (Fig. 2B); (3) a correct in-frame protein translation and the amino acid residues were identical to those of the native protein (data not shown); (4) The molecular mass was ~11,107, and there was only a single predominant peak in the MALDI-MS image (Fig. 2C).

Enhancement of GST-RADD Expression Level by Optimizing the Rare Codons in *E. coli* Rosetta (DE3)

The codon usage at the GST-RADD cassette was analyzed by the "*E. coli* Codon Usage Analysis 2.0" software. The results showed that the rare codon GGA, the 11th rarest codon in *E. coli* mRNA, was detected in the −5 position, which could result in the stalling and termination of translation [22]. To enhance the fusion protein expression, the codon GGA corresponding to glycine was changed to GGC. The engineered plasmid called pGEX-Δ1-ADAM15 (Fig. 3B) was fully checked by DNA sequencing and transformed into *E. coli*

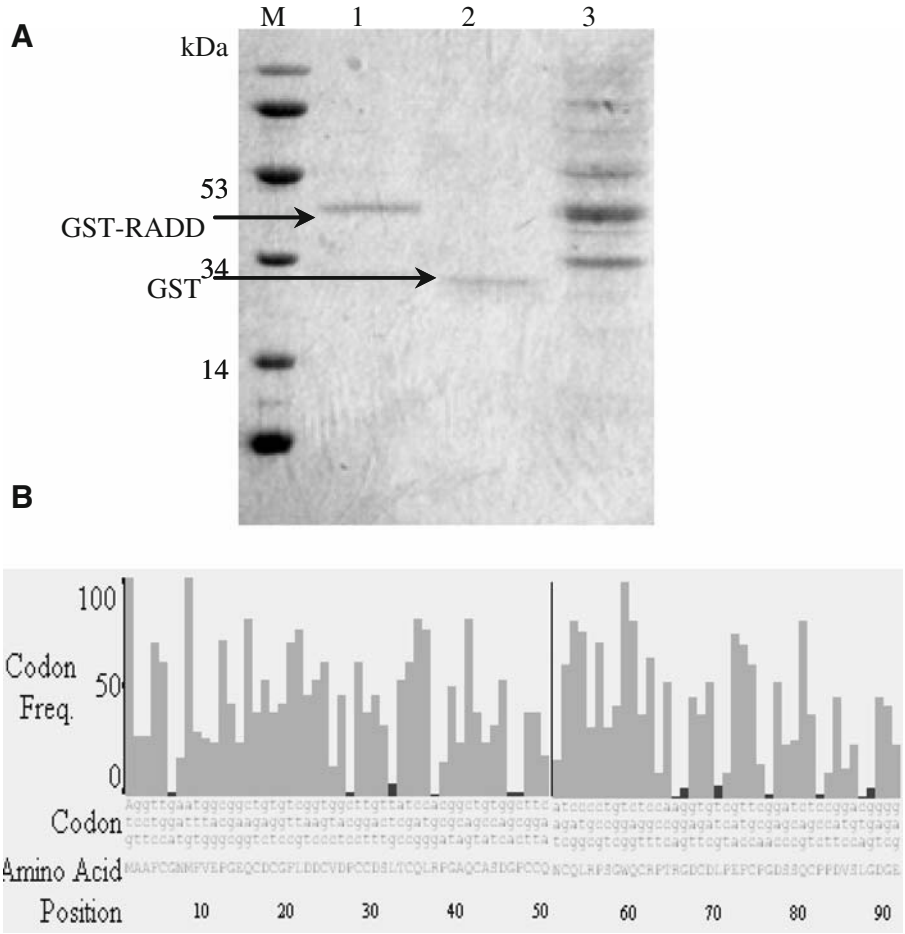

Fig. 1 (**A**) SDS-PAGE analysis of the recombinant GST-RADD expressed in the *E. coli* BL21 strain. Lane *1*, purified GST-RADD; lane *2*, GST; lane *3*, total of the cellular proteins. (**B**) Analysis of the codon usage at the human ADAM15 disintegrin domain by the "*E. coli* Codon Usage Analysis 2.0" software

Rosetta (DE3). Under the optimal induction conditions, the amount of GST-RADD protein and RADD were 9.4% (326 mg/l) and 7.1% (45 mg/l) higher than the corresponding values of the *E. coli* Rosetta (DE3) strain bearing the plasmid pGEX-ADAM15, respectively.

Enhancement of RADD Production by Improving Thrombin Cleavage Rate

The analysis of nucleotide and amino acid sequence at the GST–RADD junction demonstrated that, at the thrombin cutting site, there were three extra residues (Pro, Glu, and Phe) at the position −3, −2, and −1 from the first amino-terminal residue of RADD (Fig. 3A). In this study, the Phe, Glu and the rigid Pro residues were deleted to achieve an engineered plasmid pGEX-Δ2-ADAM15. Another engineered plasmid pGEX-Δ-ADAM15, was also obtained from the plasmid pGEX-Δ1-ADAM15 (Fig. 3B). The four plasmids pGEX-ADAM15, pGEX-Δ1-ADAM15, pGEX-Δ2-ADAM15, and pGEX-Δ-ADAM15 were then transformed into *E. coli* Rosetta (DE3) to create the corresponding

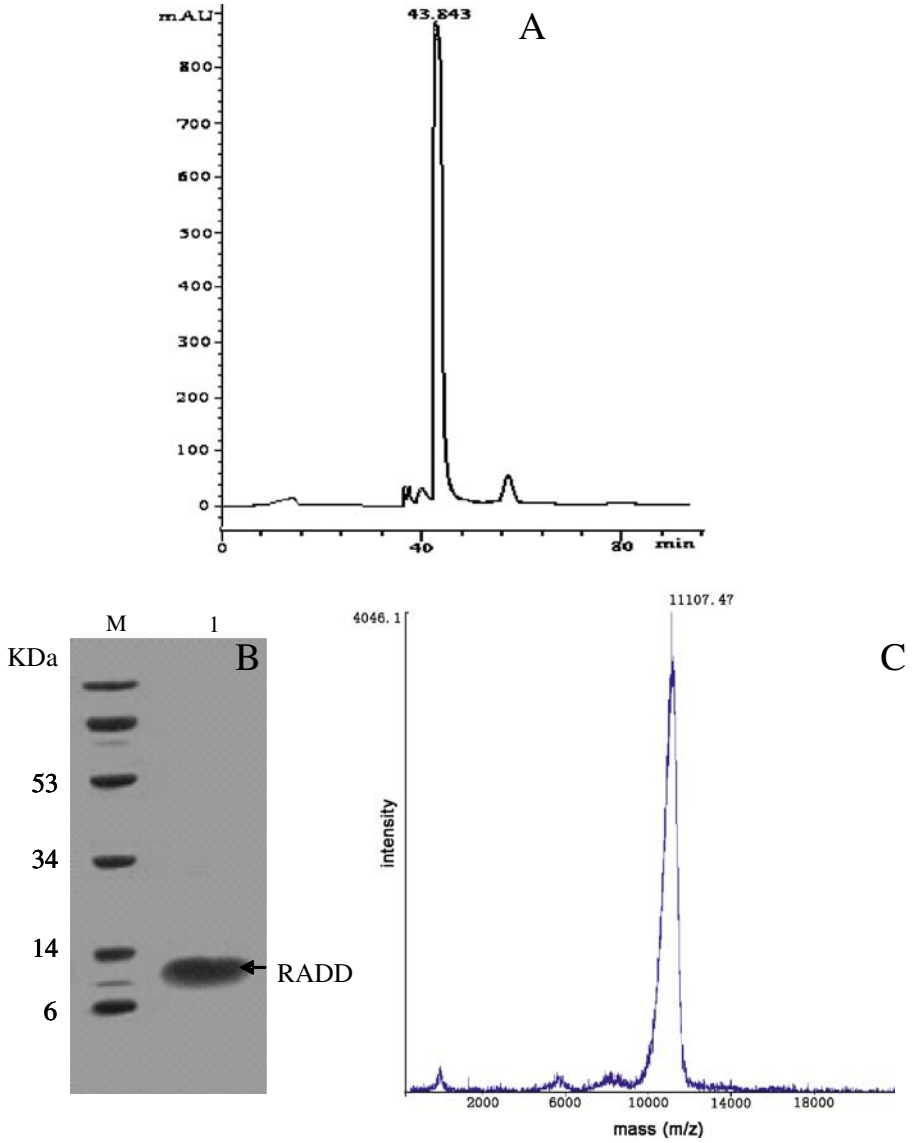

Fig. 2 (**A**) High-performance liquid chromatography assay of purified RADD with SephadexG-75. The retention time of RADD was 43.843 min. (**B**) Western blot analysis of purified RADD protein. Lane 1, RADD were detected by immunoblotting with the anti-human ADAM15 ectodomain antibody (1:1,000). (**C**) Mass spectrometry determination of purified RADD. The average mass of the large peak indicates the molecular weight of the RADD protein is 11,107.47

fusion protein GST-RADD, GST-Δ1-RADD, GST-Δ2-RADD, and GST-Δ-RADD (Fig. 4A). The following are the results of the experiment: (1) among the four fusion proteins, production of GST-Δ-RADD was the highest; (2) as illustrated in Fig. 4B, the concentrations of the purified RADD proteins were 42 mg/l (RADD), 45 mg/l (Δ1-RADD), 57 mg/l (Δ2-RADD), and 68 mg/l (Δ-RADD), respectively; (3) no any protein bands appeared in Lanes 2–4 in Fig. 4B for the lowest thrombin digest efficiency; (4) All

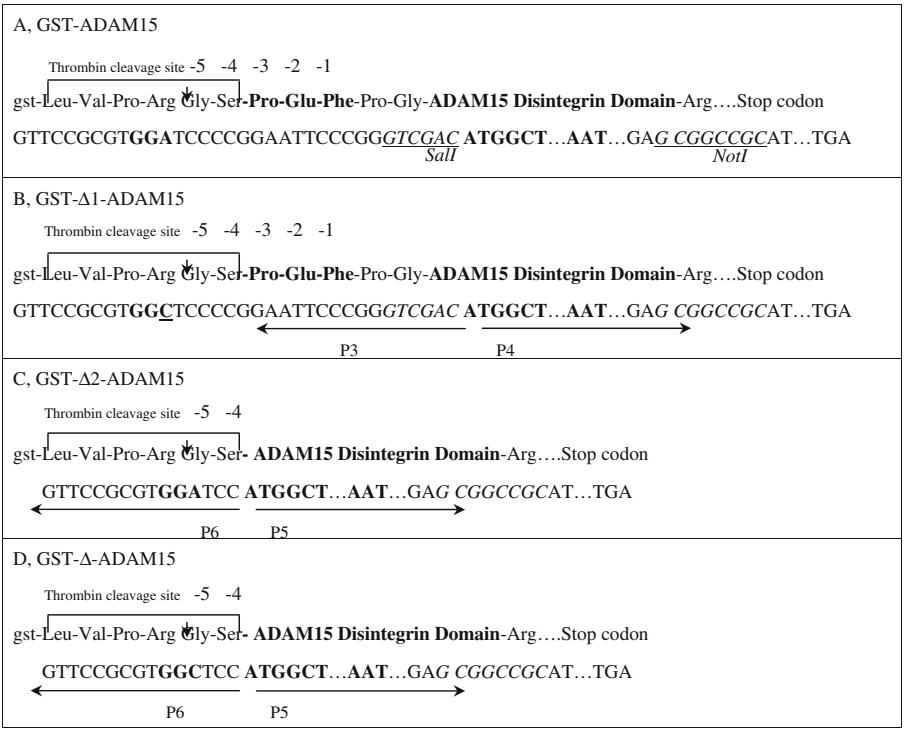

Fig. 3 Design and engineering of the fusion protein GST–RADD junction. The vertical arrow shows the thrombin cleavage site. Positions −1, −2, −3, −4, and −5 pertain to the Phe, Glu, Pro, Ser, and Gly, respectively. (**A**) Sequence of wild-type protein GST-RADD obtained by cloning ADAM15 disintegrin domain in the pGEX-4T-1 plasmid with *Sal*I and *Not*I sites. (**B**) Sequence of protein GST-Δ1-RADD obtained by optimizing rare codon GGA. P3 and P4 pertain to the mutagenic primers. (**C**) Sequence of protein GST-Δ2-RADD obtained by deleting the Pro-Glu-Phe residues. P5 and P6 are respect to the mutagenic primers. (**D**) Sequence of protein GST-Δ-RADD obtained by optimizing rare codon GGA and deleting the Pro-Glu-Phe residues. P5 and P6 pertain to the mutagenic primers

RADD proteins from the different strategies were determined to have identical amino acid sequences.

The amount of GST-RADD and of RADD protein obtained with different operational strategies are listed in Table 1. The following results could be summarized from the table: (1) the amount of GST-RADD increase by 150.4% by using *E. coli* Rosetta (DE3) as host strain, compared with that of *E. coli* BL21 (DE3) (strategy 1); (2) the amount of GST-RADD increased to 326 mg/l when GGA changed to GGC, but RADD only increased to 45 mg/l (strategy 2); (3) the amount of GST-RADD was slightly enhanced when deleting the "Pro-Glu-Phe" residues, but the RADD level increased to 57 mg/l (strategy 3); (4) by combinational utilization of strategy 2 and strategy 3, the highest amount of GST-RADD (332 mg/l) and of RADD (68 mg/l) were achieved.

RADD In Vitro Inhibited the Proliferation and Migration of HMEC-1 Cell

As indicated in Fig. 5, the biological activities of purified RADD from both *E. coli* Rosetta (DE3) and BL21 were examined on HMEC-1 cells. RADD from Rosetta (DE3) exhibited a higher inhibitory effect on the growth of HMEC-1 cells than that of the BL21 strain. The

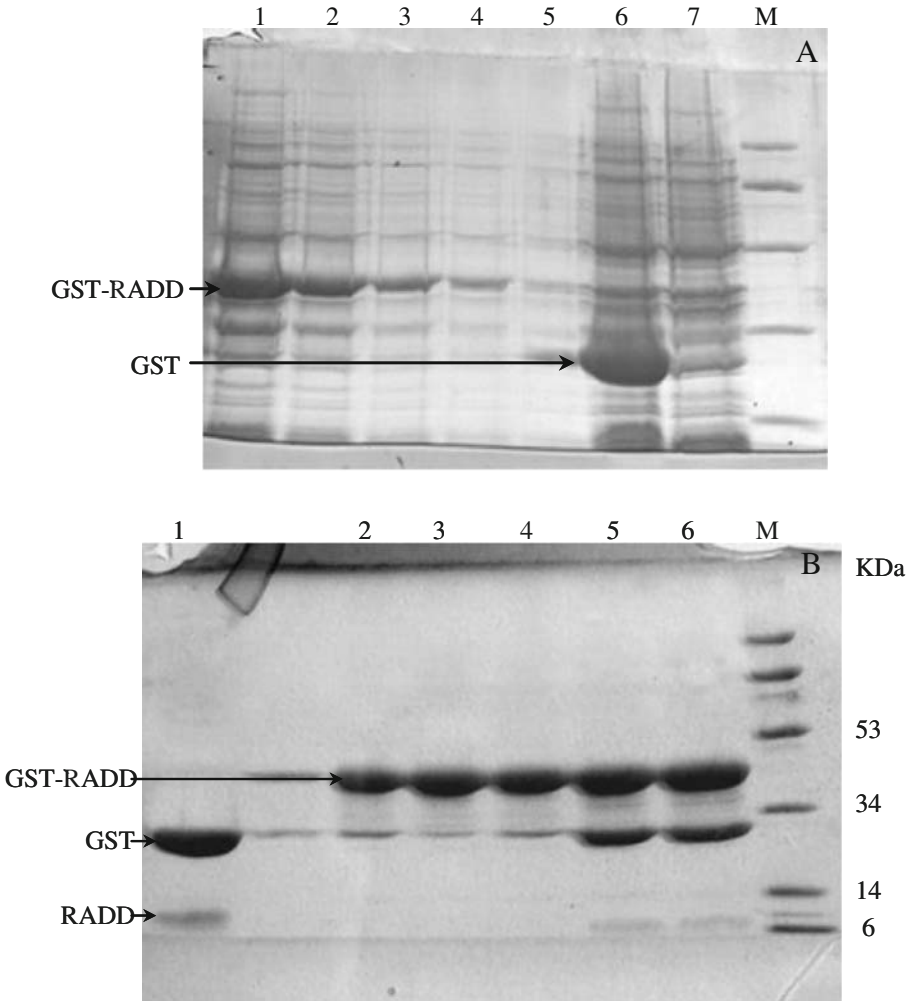

Fig. 4 (**A**) SDS-PAGE analysis of the recombinant GST-RADD expressed under different strategies. Lanes *1–4*, fusion proteins GST-Δ-RADD, GST-Δ2-RADD, GST-Δ1-RADD, and GST-RADD, expressed in Rosetta (DE3), respectively; lane *5*, GST-RADD expressed in BL21(DE3); lane *6*, the cellular proteins containing GST; lane *7*, the cellular proteins of Rosetta (DE3) without induction. (**B**) SDS-PAGE analysis of improved thrombin cleavage at the engineered GAT–RADD junction. Lane *1*, the cleavage of GST-Δ-RADD from *E. coli* Rosetta (DE3) using 10 U thrombin protease at 4°C for 8 h; lane *2*, the cleavage of GST-RADD from *E. coli* Rosetta (DE3); lanes *3* and 4, the cleavage of GST-Δ1-RADD from *E. coli* Rosetta (DE3) and BL21, respectively; lanes 5 and 6, the cleavage of GST-Δ2-RADD from *E. coli* Rosetta (DE3) and BL21, respectively

IC_{50} value for both cases was 7.0 and 20 μg/ml, respectively (Fig. 5A). In addition, RADD inhibited HMEC-1 cells migration in a time-dependent manner. The migration rate of HMEC-1 cells was 31±4.8% of the control at 72 h with the presence of 5 μg/ml RADD protein from Rosetta (DE3) strain. However, the RADD protein from BL21 strain had slightly inhibitory effect on HMEC-1 cells migration under the same conditions as shown in Fig. 5B.

Table 1 Comparison of the results with different strategies.

Plasmid	*E. coli*	GST-RADD(mg/l)[a]	RADD /(mg/l)[a]
pGEX-ADAM15	BL21	119	18
	Rosetta (DE3)	298 (0%)[b]	42 (0%)[b]
pGEX-Δ1-ADAM15 (With GGA changed to GGC)	BL21	126	18
	Rosetta (DE3)	326 (9.4%)	45 (7.1%)
pGEX-Δ2-ADAM15 (With Pro-Glu-Phe deleting)	BL21	118	21
	Rosetta (DE3)	306 (2.7%)	57(35.7%)
pGEX-Δ-ADAM15 (With GGA changed to GGC and Pro-Glu-Phe deleting)	BL21	131	24
	Rosetta (DE3)	332(11.4%)	68(61.9%)

[a] The protein concentration was determined by BCA Assay.

[b] The increasing percent was compared with that of RADD expressed in *E. coli* Rosetta (DE3) containing the plasmid pGEX-ADAM15.

Discussion

In this study, based on the analysis of the complementary DNA (cDNA) of RADD and the amino acids of the fusion protein GST-RADD cassette, three different strategies were examined to improve the production of the functional human ADAM15 disintegrin domain in *E. coli*. The amount of GST-RADD increased to 298 mg/l by directly adopted the *E. coli* Rosetta (DE3) as host strain based on the coding sequence of the targeted protein. The amount of GST-RADD further increased to 326 mg/l through changing GGA to GGC in the GST-RADD cassette. Furthermore, the highest yield of RADD (68 mg/l) achieved by removing the Pro-Glu-Phe residues at the thrombin cutting site to increase the thrombin cleavage efficiency.

In this study, with *E. coli* BL21 (DE3) as host strain, a low-level expression and poor bioactivity of RADD were observed. Similar results were also observed in *E. coli* by Jeon OH *et al*. [8]. For the reason, many reports have well demonstrated that a strong correlation exists between the frequency of codon usage and the level of its cognate tRNA, and the presence of rare codon limits the expression level and bioactivity of the heterologous target protein [14, 23–25]. Codon preference analysis was carried out in the coding sequence of human ADAM15 disintegrin domain (Fig. 1B) and the result showed that presence of the rare codons (GGA and GGG for Gly, CCC for Pro, TTG for Leu, and AGG and AGA for Arg) was 14%. To avoid the limitation of rare codon, the first strategy considered in this study was to use *E. coli* Rosetta (DE3) containing the chloramphenicol-resistant plasmid with additional copies of tRNA with rare codons (AUA, AGG, AGA, CUA, CCC, and GGA) as the host strain. As a result, 298 mg/l GST-RADD and 42 mg/l RADD were obtained.

Codon preference analysis at the junction area showed the presence of the rare glycine codon GGA was high and the usage frequency was very low (Fig. 1B). As the second strategy, the wild plasmid pGEX-ADAM15 was engineered to pGEX-Δ1-ADAM15 by optimizing the rare codon GGA to GGC in the cassette. The GST-RADD protein from *E. coli* Rosetta (DE3) bearing the plasmid pGEX-Δ1-ADAM15 increased to 72% of the total cellular proteins, which was 9.4% higher than that of the plasmid pGEX-ADAM15. However, the concentration of RADD only increased by 7.1%, compared with that of the wild plasmid case. This result implied that increasing the amount of fusion protein GST-RADD only without increasing the efficiency of thrombin digestion at the junction of GST and RADD, could probably not enhance the RADD concentration.

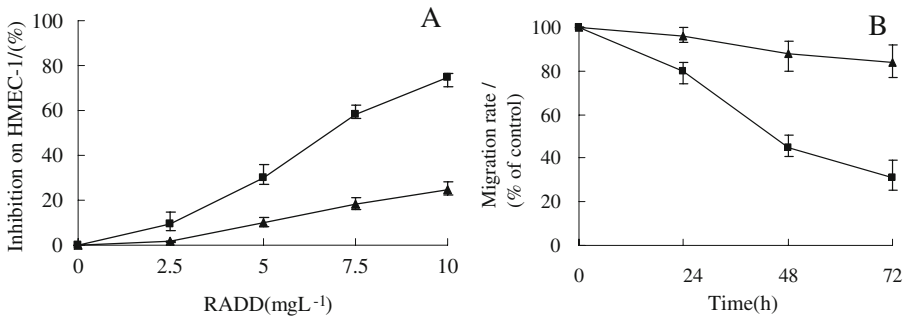

Fig. 5 The biological activity of RADD on HMEC-1 cells. *Filled square* represents RADD from *E. coli* Rosetta (DE3) and *filled triangle* was from BL21 strain. (**A**) Inhibition of RADD on HMEC-1 cell proliferation. The HMEC-1 proliferation was estimated in the presence of the RADD concentrations at 0, 2.5, 5, 7.5, and 10 μg/ml after a 72-h incubation, respectively. Control was the corresponding concentrations of GST in HMEC-1 cell culture. Results were expressed as percentages of the controls and were the means ±SE of three determinations. (**B**) Inhibition of RADD on HMEC-1 cell migration. The migration rate was defined as the migration distances of RADD-treated (5 μg/ml) cells relative to that of the control (GST) and expressed in percentage. Results were the means ±SE of three determinations at different microscopes at 0, 24, 48, and 72 h, respectively

The thrombin cleavage efficiency was the key factor for increasing RADD concentration from the fusion protein GST-RADD. The amino acid sequence analysis of wild GST–ADAM15 junction is illustrated in Fig. 3A. First, the Pro at position −3 could decrease the flexibility of the thrombin recognition site because its ring structure is conformationally restricted. Second, the Glu at the −2 position is the acidic amino acid that is unfavorable for thrombin digestion. To further increase the concentration of RADD, the third strategy was developed to improve the digestion efficiency of thrombin by deleting the Pro-Glu-Phe residues at the GST–RADD junction, and the engineered plasmids pGEX-Δ2-ADAM15 and pGEX-Δ-ADAM15 were constructed. The fusion protein GST-RADD obtained from the strain containing the two plasmids were digested under the same condition. As a result, RADD concentration reached the level of 57 mg/l and 68 mg/l, which was 35.7% and 61.9% higher that that of the control (42 mg/l) (Table 1).

The recombinant disintegrin domain of ADAM15 (Met^{420} to Glu^{510}) contained 91 amino acids, and the calculated mass was about 9,661 Da. However, in this research, the molecular mass of RADD was 11,107 Da. According to the previous publications on RADD, there are three different molecular mass of ADAM15: 12 kDa, 10 kDa, and 9,517.4± 1 Da [5, 6, 8, 11]. For the 12-kDa case, the disintegrin-like domain of ADAM15 was expressed in *P. pastoris*. For the second case, the cDNA fragment was inserted into the pGEX-2T vector and expressed in *E. coli* DH5. For the last case, RADD was achieved from *E. coli* DH5 with pGEX-3X as vector. All those results indicated that the molecular mass of ADAM15 may vary with the host strains or vectors. In this research, a 273-bp cDNA fragment was cloned into pGEX-4T-1 vector and RADD protein was expressed in *E. coli* Rosetta (DE3). In addition, ADAM15 disintegrin domain contained 15 cysteine residues with multiple disulfide bonds. The cysteine residues may exist free, or form the disulfide bond with the cysteine in the cysteine enriched domain, and the Rosetta (DE3) strain designed to express the eukaryotic protein is helpful for the formation of disulfide bonds. All of them might be the cause for the mass of RADD increasing. The detailed mechanisms causing different molecular mass of RADD were less known and waiting for further investigation.

The results presented here demonstrated that the production of recombinant protein can be significantly increased by releasing the rare codons and amino acids restriction. This is probably the first report of getting milligram of the recombinant human ADAM15 disintegrin in *E. coli* strain. In addition, increasing the production of human ADAM15 disintegrin in *E. coli* by releasing the rare codons and amino acids restriction may provide an alternative approach to enhance other recombinant proteins production with *E. coli*.

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