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Improvement of Artificial Antibody Secretion Using Supercharged Protein

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A repebody, an artificial non-immunoglobulin protein scaffold, is expected to be a solution in the search for faster, cheaper, and customizable antibodies. However, the production of medical repebodies remains difficult due to their low yield and the complex purification processes required. The *Pseudomonas fluorescens* ABC transporter system has been suggested as an efficient and cost-effective method for repebody production, but the total yield is low because of the secreted protein's positive charge; thus, a repebody with a high isoelectric point needs to be changed into a more negatively charged protein for better secretion. To achieve this, we first attached oligo-aspartic acids to the N- and C-terminals of the repebody, but secretion efficiency was not enhanced significantly. Subsequently, we devised an alternative method for improved secretion efficiency by engineering fifteen positively charged amino acids to aspartic acid in the non-antigen binding sites of the repebody to give a high net negative charge. As a result, secretion efficiency was greatly enhanced from 21.2% (wildtype) to 58.5% (negatively supercharged). The negatively supercharged repebody was successfully produced extracellularly by ABC transporter secretion system in *P. fluorescens*.

Key words : ABC transporter, Isoelectric point (pI), negatively supercharged, *Pseudomonas fluorescens,* repebody

Introduction

Variable Lymphocyte Receptors (VLRs) are antigen receptors that exist in jawless fish. Jawless fish recognize antigens by Leucine-Rich Repeats (LRRs) which form the basis of VLRs instead of the immunoglobulin fold that all vertebrates use in their adaptive immune system. VLRs are the only natural adaptive immune system which produces proteins that do not have Ig-folds and can theoretically recognize almost all antigens like antibodies [12].

Immunoglobulin antibodies are relatively difficult and costly to manufacture. On the other hand, VLRs are easier to engineer or mutate compared to IgG whose total molecular weight is about 150 kDa and is highly glycosylated while VLRs are less than 40 kDa and does not undergo glycosylation [19]. In addition, VLRs can recognize antigens even with only a single chain. It is also a stable protein that remains functional at room temperature for more than a month [5]. It also has been shown that VLRs can be mass-produced using *Escherichia coli* [20]. All these suggest that VLRs are a suitable alternative to immunoglobulin antibodies.

Repebody is named after the repetitive modules that make up VLRs and its function as an antibody in nature. The repebody scaffold can be engineered into artificial antibody mutating LRRs by module engineering [11]. Its ability to bind to various antigens depending on the amino acid sequence of its antigen binding site also allows it to be developed as a custom antibody [11]. Moreover, several studies involving repebody have shown its feasibility. Repebody that is modulated to have binding affinity for human Interlukin-6 (hIL-6) is highly specific to hIL-6 and can remarkably suppress growth of tumors by locking hIL-6/STAT3 signaling [10]. The developed anti-VEGF repebody, anti-human C5a-repebody and anti-human EGFR-repebody have also shown high specificity to VEGF, C5a or EGFR and block VEGF, C5a or EGFR related cell signaling processes and can suppress diseases both *in vitro* and *in vivo* [6, 7,

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10, 22]. Given the ease of designing and engineering repebodies as well as its high stability in high temperatures and pH, repebodies offer a desirable alternative to high custom antibodies.

In order to commercialize repebodies, a mass production system has to be well established. Currently it is difficult to purify these proteins because of the large amount of associated cells that has to be broken down due to the fact that they remain inside E. coli after production. Furthermore, contamination by LPS, a toxic material of *E. coli*, is inevitable when the cells are broken down. These problems can be resolved by secreting proteins to the extracellular medium, reducing purification steps and cost.

Here, we tried to produce repebody using an ABC transporter composed of TliD, TliE and TliF, where TliD is an ATP Binding Cassette (ABC), TliE is a Membrane Fusion Protein (MFP) and TliF is an Outer Membrane Protein (OMP). The ABC transporter is a Type 1 Secretion System (T1SS). Compared with the other secretion systems, T1SS is structurally simple and suitable for use in a Protein Manufacturing Factory (PMF) since the proteins are secreted directly to the extracellular medium from the cytoplasm and can produced in a continuous culture because it is not necessary to lyse the cells [15]. Using *P. fluorescens* equipped with an ABC transporter, we were able to produce repebody in a reusable and eco-friendly way. *P. fluorescens* has several advantageous features for recombinant protein production such as its safety, adequacy for high cell density culture and export system [8, 14]. In addition, many recombinant proteins conjugated with Lipase ABC transporter Recognition Domain 3 (LARD3) of thermostable lipase TliA secreted by TliDEF, were well-expressed and secreted in a developed strain of *P. fluorescens ΔtliAΔprtA* in previous studies [13, 17]. Therefore, *P. fluorescens* is useful as an expression host for various recombinant proteins.

However, despite such favorable features, the low secretion efficiency of repebody remains a problem. In previous studies, it was found that the key factor to determine protein secretion by the TliDEF ABC transporter is the pI value of the target protein [3]. It was shown that negatively supercharging proteins increased secretion efficiency by the ABC transporter. In this paper, we designed a negatively supercharged repebody and constructed a more efficient mass production system using the ABC transporter.

Materials and Methods

Construction of plasmid vectors with inserted target genes

Three kinds of shuttle plasmid vectors which can be used in *E. coli* and *P. fluorescens* were used: pDART, pBD10 and pFD10 [3]. The pDART vector contains genes coding for *tliD*, *tliE*, *tliF*, Lipase ABC transporter Recognition Domain 3 (LARD3) and various restriction enzyme sites for target gene insertion [16]. pFD10 has aspartic acids upstream of the cloning site and pBD10 has aspartic acids downstream of the cloning site [3]. Different repebodies were cloned into XbaI-SacI of the plasmid vectors via PCR using plasmid shown in Table 1.

Design of negatively supercharged repebody

The negatively supercharged repebody was designed by mutating some amino acids into aspartic acid. The decision of mutating amino acids into aspartic acid stems from the fact that aspartic acid (D) is better than glutamic acid (E) at lowering pI value. The repebody scaffold has concave and convex regions. The concave region is the antigen-binding site, thus associated amino acid sequences were not mutated to maintain antigen affinity. Therefore, only positive amino acids in the convex region were selected and mutated into aspartic acid (Fig. 4B).

Protein expression

Plasmid construction was performed in *E. coli* XL1-Blue, while protein expression and secretion were performed in *P. fluorescens ΔtliAΔprtA*, which is a double-deletion mutant of *P. fluorescens* SIK-W1 [3]. Cloned genes (Table 1) were transformed into *E.coli* XL1-Blue and incubated at 37℃ in

Table 1. Oligonucleotide primers used for DNA inserts in plasmid construction

	Primer name	Sequence (5′-3′)
pDART-,pFD-, pBD-repebody-3, repebody-5, repebody-6, repebody (-)	XbaI-repebody	GGGTCTAGAGAAACCATTACCGTGAGCAC
	XbaI-H_6repebody	GGGTCTAGACATCACCATCACCATCACGAAACCATTACCGTGAGCAC
	repebody-SacI	GGGGAGCTCGGTCGGGCAATAATGCTAC
pDART-repebody (+)	XbaI-repebody(+)	GGGTCTAGAGAAACTATCACCGTCTCTACA
	repebody(+)-SacI	GGGGAGCTCGGTCGGACAAATGCTACG

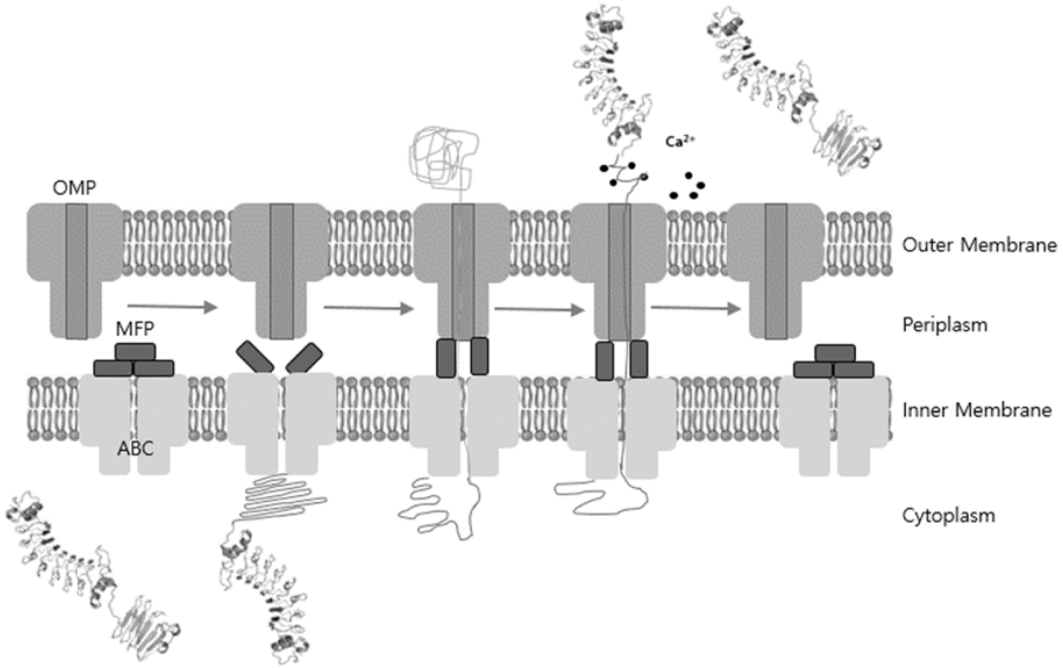

Fig. 1. Protein secretion using ABC transporter system. The outer membrane protein [18], ABC protein (ABC), and membrane fusion protein (MFP) are separate from each other in the resting state. Binding of the C-terminus of LARD3 to the ABC protein causes structural changes in the ABC protein that leads to the assembly of a T1SS. The target protein is then secreted into the extracellular medium through the ABC transporter. Extracellular calcium ions attach to certain parts of the signal sequence and pull the remainder of the protein out of the cell. After the protein is completely secreted, the three proteins of the secretion system separate again and wait for another protein to bind to.

LB medium. Kanamycin was the antibiotic used at a concentration of 30 μg/ml. To verify protein expression, plasmids with inserted target genes were transformed into *P. fluorescens ΔtliAΔprtA* via electroporation at 2.5 kV, 125 Ω and 50 μF. Afterwards, it was cultured at 25℃ in a Terrific Broth (TB) medium containing kanamycin (60 μg/ml), it was placed in a 180 rpm shaking incubator until it reached the stationary phase. The TB medium consisted of 1.2% pancreatic digest of casein, 2.4% yeast extract, 17 mM KH_2PO_4, 72 mM K_2HPO_4 and 0.4% glycerol.

Analysis of repebody expression

Recombinant cells were grown in a TB medium supplemented with 60 μg/ml kanamycin. The proteins of the cell pellet and supernatant were analyzed using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gels, following the method developed by Laemmli [4]. The proteins were transferred onto a nitrocellulose membrane (Amersham, UK) for western blot, which was performed as previously described using anti-LARD3 as the primary antibody and anti-rabbit IgG as the secondary antibody chemiluminescence system (Advansta, USA). Detection was performed using Azure C600 automatic detection system.

Protein purification

The harvested broth underwent centrifugation at 4,000 rpm for 30 minutes, and the clear supernatant was purified through affinity chromatography using a Ni-NTA column (HisPur^{TM} Ni-NTA Resin) (Thermoscientific, USA). The column was equilibrated with an equilibration buffer of pH 7.4 containing 20 mM sodium phosphate and 200 mM NaCl. During the binding step, NaCl concentration was maintained at 200 mM. Bound proteins were washed with washing buffer of pH 7.4 containing 200 mM NaCl and 10 mM imidazole. This was followed by elution with an elution buffer containing 250 mM imidazole. Each sample was loaded into lanes of 4-12% polyacrylamide gradient gel (Bolt 4-12% Bis-Tris Plus) (Invitrogen, USA). Then, we stained the gel with Sun gel staining solution (LPS solution, South Korea).

Computational analysis

The theoretical pI values of the target proteins were calculated using the ExPASy Compute pI/Mw tool [1, 2, 21]. The

pI value was calculated based on the protein sequence, including the LARD3 sequence. For the tertiary structure study, SWISS MODEL structural homology modelling was used (https://swissmodel.expasy.org).

Results

Measurement of original repebodies secretion efficiency

The repebodies used in this article were repebody-3, repebody-5 and repebody-6. Each repebody has a different number of Leucine-Rich Repeats (LRRs) - 3, 5, 6 for each - which is one of the repebody modules. The repebodies were expressed in *P. fluorescens ΔtliAΔprtA* and secreted to the media through the TliDEF ABC transporter. Results were quantitatively analyzed using western blotting (Table 2). Secretion was limited with only 10-20% of the repebody being secreted while 80-90% remained in the cell (Fig. 2).

Addition of negatively-charged oligo amino acids

According to previous research [3], secretion efficiency can be improved by attaching negative amino acids to the protein. In order to make the repebody more negatively charged, we tagged 10 aspartic acids (Asp) on to the repebody by inserting the repebody-5 gene (referred to as repebody) into plasmids which had codons for 10 Asps. Two different vectors were used, one with aspartic acids upstream of the repebody (pFD10-repebody) and the other with aspartic acids downstream of the repebody (pBD10-repebody). The Asp_{10}-repebody (FD) and repebody-Asp_{10} (BD) were compared to the normal repebody (DART). The properties of Asp-tagged repebodies are shown in Table 3. The 10 aspartates attached decreased the estimated pI of the repebody from 6.1 to 4.7. However, since the increase in secretion efficiency was insignificant in spite of the lower overall pI value (Fig. 3), we tried to find another way to change the charge of the repebody.

Table 2. Properties and secretion efficiency of wild type repebodies

Proteins	repebody-3	repebody-5	repebody-6
pI	5.9	5.8	5.8
MW (kDa)	36.7	42.1	44.8
Cell (ng/μl)	7.2	2.3	2.5
Supernatant (ng/μl)	0.8	0.5	0.6
Efficiency (%)[a]	14.9±6.4	17.2±6.6	19.4±5.9

[a]The amount of protein produced in the cell and supernatant was estimated from the densitometry of reference protein. The secretion efficiency was calculated as the amount of secreted protein divided by the total of produced protein; supernatant / (cell + supernatant)×100.

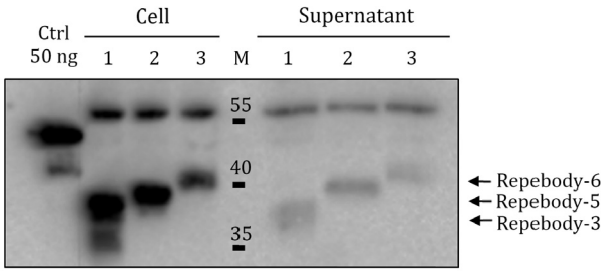

Fig. 2. Secretion of repebodies. Western blot analysis was performed to check repebody secretion by *P. fluorescens* using an anti-LARD3 primary antibody. Three different pDART plasmids, each containing the gene for one of repebody-3, repebody-5, and repebody-6, were transformed into *P. fluorescens ΔtliAΔprtA* and the colonies were screened from LB agar plates containing 30 μg/ml kanamycin. The *P. fluorescens* was then cultured in TB medium at 25℃. For comparison, equivalent amounts of cell extract and culture supernatant (16 μl) were loaded onto the gel. A 50 ng control sample was loaded as a reference. The TliDEF ABC transporter was able to secrete repebody-3, repebody-5, and repebody-6. However, the majority of the repebodies remained inside the cell (labelled "C") and only a small percentage was found in the supernatant (labelled "S). 1, repebody-3 (36.7 kDa); 2, repebody-5 (42.1 kDa); 3, repebody-6 (44.8 kDa); M, Size Marker.

Table 3. Properties and secretion efficiency of oligo aspartate-fused repebodies

Vectors	pDART-repebody	pBD-repebody	pFD-repebody
pI	5.3	4.7	4.7
MW	41.3	42.5	42.5
Cell (ng/μl)	1.3	3.3	1.1
Supernatant (ng/μl)	0.2	0.8	0.3
Efficiency (%)[a]	15.0±8.9	20.0±5.8	23.3±0.9

[a]The amount of protein produced in the cell and supernatant was estimated from the densitometry of reference protein. The secretion efficiency was calculated as the amount of secreted protein divided by the total of produced protein; supernatant / (cell + supernatant)×100.

Design of negatively charged repebody

Instead of inserting negatively-charged oligomeric peptides, we mutated amino acids found throughout the sequence of the original repebody. This method is based on the fact that hydrophilic amino acids exposed outside the

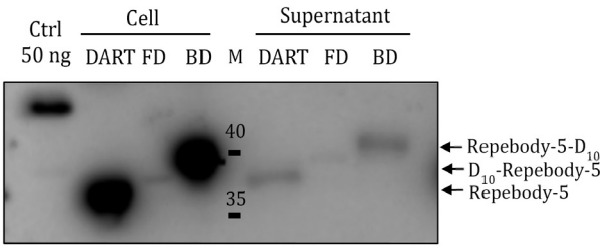

Fig. 3. Secretion of repebodies tagged with ten aspartates. Western blot analysis was performed to check repebody secretion by *P. fluorescens* using an anti-LARD3 primary antibody. The repebody-5 gene was integrated into three different plasmids: pDART, pFD10, and pBD10. pFD had ten aspartates attached upstream of the repebody-5 gene, pBD10 had ten aspartates attached downstream of the repebody-5 gene, while pDART had no aspartate oligopeptide attached to it. The three different plasmids were then transformed into *P. fluorescens ΔtliAΔprtA* and colonies were screened from LB agar plates containing 30 μg/ml kanamycin. The *P. fluorescens* was cultured in TB medium at 25℃. For comparison, equivalent amounts of cell extract and culture supernatant (16 μl) were loaded onto the gel. A 50 ng control sample was loaded as a reference. The protein band of pBD10-repebody can be seen both in the cell lane (labelled "C") and supernatant lane (labelled "S") but it was slightly shifted upwards. On the other hand, the protein band of pFD10-repebody can be seen in both the cell and supernatant lanes but is significantly fainter compared to pDART-repebody and pBD10-repebody. DART, pDART-repebody; FD, pFD10-repebody; BD, pBD10-repebody; M, Size Marker.

protein can be changed into different hydrophilic amino acids provided that they do not interact with other amino acids in the protein. By changing the charge of the protein, the method has been found to increase the solubility of the protein [18]. Amino acids were carefully selected so as not to significantly affect the structure, then these amino acids were mutated into negatively charged amino acids. The resulting sequence is listed in Fig. 4A. By SWISS modeling, it was shown that no significant structural change had occurred. (Fig. 4B) As a result, the estimated net charge at pH 7 changed from -1.2 to -22.4 and the pI value of the repebody decreased from 5.8 to 4.2. Using this method, we could design and produce a negatively charged repebody (repebody (-)). In addition, we also constructed a positively charged repebody (repebody (+)) in which some amino acids in the convex region of the wild type repebody were replaced with Lysine (K), a positively charged amino acid. The properties of repebodies with different supercharges are shown in Table 4. Western blotting results show that repebody (+) was not secreted at all while repebody (-) showed higher secretion efficiency (ratio of secreted versus intracellular protein) than the wild type repebody. Secretion efficiency increased from 21.2% to 58.5% in repebody (-) (Fig. 5).

Table 4. Properties and secretion efficiency of positive and negative supercharged repebodies

Proteins	repebody (+)	repebody	repebody (-)
pI	9.6	5.8	4.2
MW	41.5	42.1	41.3
Cell (ng/μl)	5.8	7.8	1.6
Supernatant (ng/μl)	0	1.9	1.2
Efficiency (%)[a]	0±0	21.2±6.1	58.5±6.4

[a]The amount of protein produced in the cell and supernatant was estimated from the densitometry of reference protein. The secretion efficiency was calculated as the amount of secreted protein divided by the total produced protein; supernatant / (cell + supernatant) ×100.

Protein Purification

To produce repebody (-) protein, repebody (-) was expressed in *P. fluorescens ΔtliAΔprtA* in 300 ml of TB medium at 25℃. We purified a high concentration of repebody (-) using a Ni-NTA column (Fig. 6).

Discussion

In this study, we developed a repebody with increased secretion efficiency via negatively supercharging proteins. In a previous study, the pI values of the recombinant proteins were lowered by attaching 10 Asp to the proteins and this enabled them to be secreted to an extracellular medium [3]. Similarly, we tried to improve secretion efficiency by attaching 10 Asp to a repebody, however, this approach failed to increase secretion efficiency. In the case when oligo-Asp was attached to the N-terminal of the repebody (pFD10-repebody), the secretion decreased compared to a wild type repebody. This result seems to be caused by either a reduction in mRNA stability or translation rate due to alterations in the secondary structure, or due to reduced protein half-life as reported previously [3].

Accordingly, we developed another method to decrease the net charge of the repebody by replacing positively charged amino acids with negatively charged amino acids. Based on the structural modeling results, we confirmed that the amino acid substitutions did not modify the theoretical structure of the repebody. As a result of negative super-

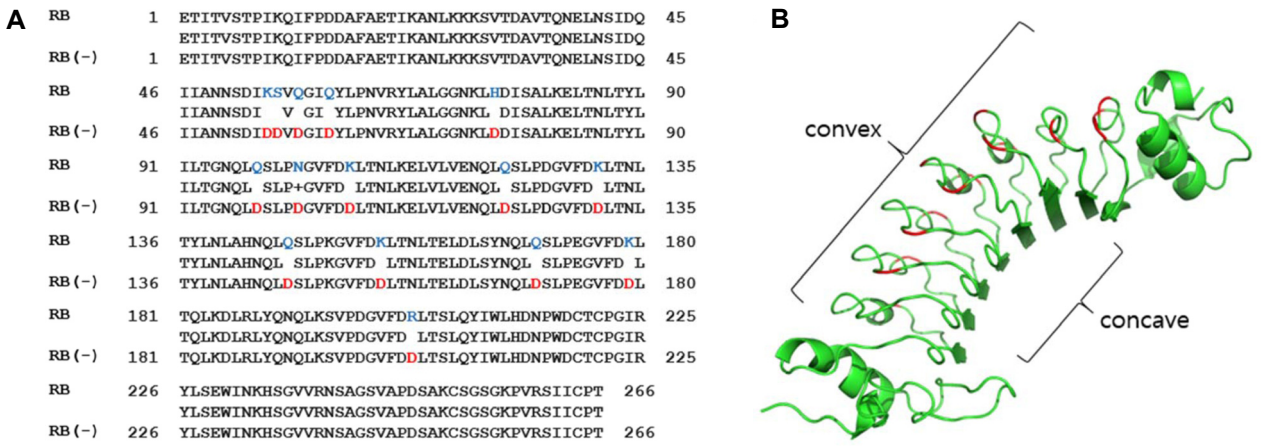

Fig. 4. Design of repebody (-). (A) Figure shows an alignment of the amino acid sequences of the wildtype repebody and repebody (-). Specific positively charged amino acids (shown in blue) were manually substituted for aspartate (shown in red). (B) SWISS 3D model of repebody (-). The residues in red indicate where the amino acid sequences were changed into aspartate. Only residues in the convex region were selected.

charging, the secretion rate was highly increased. Supercharged GFP (-36) has already been seen to secrete well without destroying protein folding or function [3, 9]. For further study, we need to carry out various experiments to validate the structure and function of the modified repebody for future commercial use. In addition, this method can be applied to other proteins to effectively increase production efficiency.

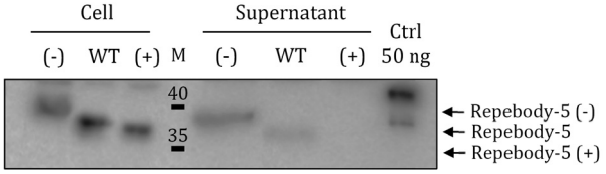

Fig. 5. Secretion of repebody (-). Western blot analysis was performed to check the secretion of repebodies of different charges by *P. fluorescens using an anti-LARD3 primary antibody*. The repebody-5 gene was positively (+) and negatively (-) supercharged. The *P. fluorescens* was cultured in TB medium at 25℃. Although both the negatively and positively supercharged repebody proteins had similar molecular weights with the wild type repebody, they showed different band locations on the membrane. For the comparison, equivalent amounts of cell extract and culture supernatant (16 μl) were loaded onto the gel. A 50 ng control sample was loaded as a reference. The protein bands in the cell (labelled "C") and supernatant (labelled "S") lane of the negatively supercharged repebody-5 was slightly shifted upwards. On the other hand, the protein band in the cell lane of the positively supercharged repebody-5 was slightly shifted downwards and no protein band can be seen in the supernatant lane. (-), repebody (-); WT, WT repebody; (+), repebody (+); M, Size Marker.

The repebodies have been developed in order to have highly specific affinity to various target proteins. Its potential as a therapeutic antibody capable of blocking related cell signaling pathways by binding to target proteins as well as

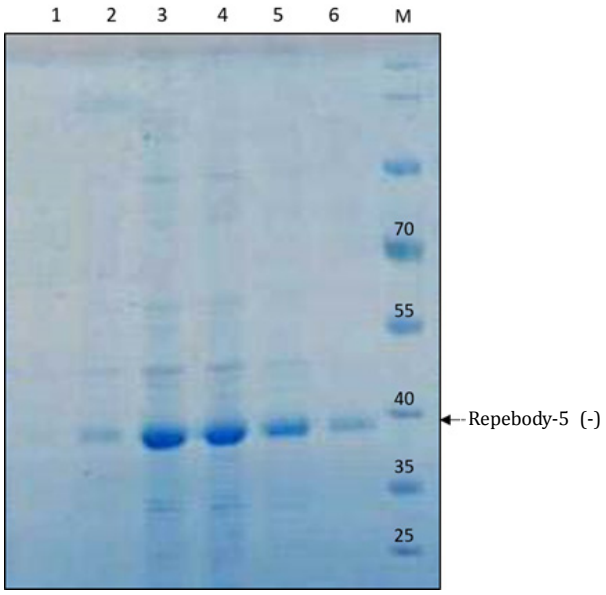

Fig. 6. Purification of repebody (-). After separating out the supernatant from the harvested broth by centrifugation, the supernatant was run through a Ni-NTA column for purification of repebody proteins via His-tag binding. The results were analyzed by SDS-PAGE. Lane1, Elution 1; Lane 2, Elution 2; Lane 3, Elution 3; Lane 4, Elution 4; Lane 5, Elution 5; Lane 6, Elution 6; M, size marker. Elution 3 (E3) and Elution 4 (E4) shows high concentration of purified H_6repebody. The size of the purified protein is indicated by an arrow (41.3 kDa).

its ability to reduce related diseases has been demonstrated extensively [6, 7, 10, 22]. The repebody used in our research is a wild type repebody lacking any biochemical activity. In later experiments, we plan to check whether the negatively supercharged repebody retained the same activity as the original one. If the negatively supercharged repebody is shown to have the same activity as the wild type, it will become a viable alternative for intracellular production of repebody in *E. coli*.

In conclusion, we successfully designed and produced negatively supercharged repebody. Repebody (-) was expressed in *P. fluorescens* and secreted through an ABC transporter system, where it displayed a vastly improved secretion efficiency. The negatively supercharged repebody was analyzed and found to have a similar structure to the original repebody based on SWISS modeling.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 단백질의 과전하화를 이용한 인공 항체의 분비 개선

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Repebody는 비면역 글로블린 인공 항체로 저렴하고 빠르게 생산 가능한 맞춤형 항체이다. 그러나 의료용 repebody의 생산은 저수율 및 복잡한 정제 공정으로 인해 여전히 어려움을 겪고 있다. *Pseudomonas fluorescens*의 ABC transporter를 사용한다면 생산 공정을 간소화하고 비용을 줄일 수는 있지만 repebody는 양전하를 띠어 분비 효율이 낮다. 따라서 등전점(pI)이 높은 repebody의 등전점을 낮추어 음전하를 띄도록 해야 한다. 이것을 위해 repebody의 N 말단과 C 말단에 연속된 아스파탐산을 붙여 보았지만 분비가 증가하지 않았다. 다른 방법으로 ABC transporter를 통한 repebody 분비 효율을 높이기 위해 repebody의 항원 결합 부위의 반대쪽에 존재하는 열다섯 개의 양전하 아미노산을 아스파탐산으로 변환하여 repebody 표면이 강한 음전하를 띠도록 하였다. 그 결과, 기존 repebody의 발현 단백질 당 분비효율은 21.2%였으나 변형한 과음전하 repebody의 분비효율은 58.5%로 향상되었다. 결론적으로 과음전하를 통해 만들어진 repebody는 *P. fluorescens*에 의해 세포 바깥에 분비 생산할 수 있었다.