Selection of a Single Chain Variable Fragment Antibody (scFv) against Subtilisin BRC and its Interaction with Subtilisin BRC

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Abstract: Background: The focus of this study was the selection of a single chain variable fragment antibody (scFv) against subtilisin BRC, a fibrinolytic enzyme using phage display, and to characterize the interaction between the antibody and subtilisin BRC.

Methods: The subtilisin BRC-specific phage clones were selected using Griffin.1 scFv phage library and sequenced. The gene of subtilisin BRC-specific scFv (scFv-BRC) from selected phage clone was expressed in E. coli and scFv-BRC was characterized. Molecular modeling of the three-dimensional (3D) structures of scFv-BRC was performed using MODELLER 9.19 modeling software and assessed by PROCHECK. Molecular docking of subtilisin BRC with scFv-BRC was carried out using PATCHDOCK.

Results: The size of scFv-BRC gene is 635bp and it consists of 54bp of heavy chain region (VH), 336bp of light chain region (VL), 45bp of a linker. scFv-BRC was actively expressed by E. coli expression vector pET28a-scFv in E. coli BL21 (DE3), and the amount of expressed scFv-BRC was about 50 mg/L. Its molecular weight is ~26kDa. The CDR domain of scFv-BRC consists of 6 amino acids in CDR L1, 3 amino acids in CDR L2 and 9 amino acids in CDR L3. Docking results of subtilisin BRC and scFv-BRC showed global energy of - 56.29 kJ/mol. Furthermore, the results showed that amino acid residues in subtilisin BRC for binding with scFv-BRC are Tyr6, Ser182, Ser204, and Gln206.

Conclusion: scFv against subtilisin BRC selected using phage display showed relatively strong binding energy with subtilisin BRC. The amino acid residues in subtilisin BRC for binding with scFv-BRC are not relevant to that in subtilisin BRC for binding with its substrates. These results suggested that scFv-BRC can be used as a ligand for detection and affinity purification of subtilisin BRC.

Keywords: Subtilisin BRC, fibrinolytic enzyme, phage display, cloning, homology modeling, molecular docking.

1. INTRODUCTION

In recent years, cardiovascular diseases (CVDs) have been a major cause of death worldwide. Vein thromboses are one kind of CVDs, which inevitably lead to a high death rate owing to their origin from the disorders of the heart and blood vessels. For example, deep vein thromboses and pulmonary embolisms can be dislodged and move to the heart and lungs, which present a threat to health. It has been reported that thrombotic embolisms are responsible for the death of more than 26 million people worldwide annually [1]. In addition, the death rate may rise in the future because of the increasingly unhealthy eating and living habits. Therefore, the discovery of effective treatments for thrombotic diseases is important to decrease the morbidity and mortality associated with thrombosis.

The underlying pathophysiological process in thrombosis is the formation of a fibrin clot by the proteolytic action of thrombin on fibrinogen. Accumulation of fibrin in the blood vessels usually leads to thrombosis, which results in myocardial infarction and other cardiovascular diseases [2]. The fibrin fiber is hydrolyzed into fibrin degradation products by plasmin, which is generated by plasminogen. The basis of fibrinolytic therapy is the administration of an exogenous plasminogen activator or fibrinolytic agents, which lyse the thrombus and restores blood flow [3].
A variety of fibrinolytic enzymes such as tissue plasminogen activator (t-PA, EC 3.4.21.68), urokinase (u-PA, 3.4.21.73), and bacterial plasminogen activator streptokinase (EC 3.4.24.29) have been extensively investigated and used as thrombolytic agents [4]. However, these enzymes are expensive, and patients may suffer from undesirable side effects such as gastrointestinal bleeding, allergic reactions [4]. The drawbacks of available drugs underscore the need to develop and exploit novel biochemical attributes of safer, direct-acting fibrinolytic proteases [2]. Strong fibrinolytic enzymes from Bacillus sp., such as subtilisin NAT [5], subtilisin J [6] and subtilisin E (EC 3.4.21.62) [7], have been purified and characterized for the development of superior thrombolytic drugs.

In D.P.R. of Korea, the subtilisin BRC derived from the traditional health food has been developed and used as a thrombolytic agent. The subtilisin BRC produced from Bacillus subtilis netto-89 is 27 kD of thrombolytic enzyme composed of 275 amino acids. Unlike other thrombolytic agents, subtilisin BRC is an effective thrombolytic agent because of its multiple functions such as fibrinolytic, elastinolytic and antioxidative activity [8].

In order to identify the characteristics, including the effectiveness and toxicity of subtilisin BRC in vivo, it is necessary to measure it quickly and quantitatively in blood. Phage display is a molecular diversity technology that allows the presentation of large peptide and protein libraries on the surface of filamentous phage. Phage display libraries permit the selection of peptides and proteins, including antibodies, with high affinity and specificity for almost any target. Phage display facilitates engineering of antibodies with regard to their size, valency, affinity, and effector functions. The selection of antibodies and peptides from libraries displayed on the surface of filamentous phage has proven significant for routine isolation and detection of peptides and antibodies for diagnostic and therapeutic applications [9].

At present, the studies on subtilisin BRC are mainly focused on the selection of high-yield strains and the optimization of fermentation parameters. However, very few studies on the selection of single-chain variable fragment antibody (scFv) against subtilisin BRC have been reported. In this study, we have selected the subtilisin BRC-specific scFv using phage display and investigated their interaction using a docking method.

2. MATERIALS AND METHODS

2.1. Bacterial Strains, Plasmids, and Reagents

The bacterial strains and plasmids used in this work are listed in Table 1. Unless otherwise stated, cells are grown at 37°C in TY broth (1.6% tryptone, 1% yeast extract, 0.5% NaCl, 1% glucose) with shaking. When required, media were supplemented with 100μg/mL ampicillin (Amp) or 50μg/mL kanamycin (Kan).

Restriction endonuclease, DNA ladder, and protein maker were purchased from Takara Biotechnology Co., Dalian. T4 DNA ligase and LA-Taq polymerase were purchased from Promega.

2.2. Panning of Phage Display Library

Griffin.1 scFv phage library (MRC Laboratories, Cambridge, UK) was used for the selection of specific binders against subtilisin BRC. The panning procedure was carried out by immobilizing subtilisin BRC (100μg/mL) overnight onto immuno tubes (Nunc, Roskilde, Denmark), which were washed with PBS buffer, blocked with 2% BSA in PBS (w/v), and incubated with phage suspension (~10^{12} cfu) at room temperature. Phage particles with the affinity for the subtilisin BRC were eluted using 50μg/mL trypsin and amplified in exponentially growing E. coli TG1 cells (Pharmacia Biotech). The panning procedure was repeated for 4 rounds and the total eluted phage titer was determined after each round.

2.3. Phage-ELISA and Sequencing Analysis

After the fifth round of panning, 50 clones were randomly selected for phage ELISA. The clones were cultured in 2×TY medium [10] and infected with M13KO7 helper phages (Pharmacia Biotech). The rescued phages were applied for phage-ELISA analyses against 100μg/mL subtilisin BRC as described previously [11]. Phage clones with

<table>
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<th>Table 1.</th>
<th>Bacterial strains and plasmid used in this work.</th>
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<tr>
<td><strong>Strains and Plasmid</strong></td>
<td><strong>-</strong></td>
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<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td>E. coli TG1</td>
<td>supE thi-1 A(lac-proAB) A(mcrB-hsdSM5) (rE mBl )</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>supE44 ΔlacU169 (p80 lacZ ΔM15) hsdR17 recA1 endA1 gyr496 thi-1 relA1</td>
</tr>
<tr>
<td>E. coli BL21 (DE3)</td>
<td>hsdSgal (Δ lacZ 857 ind1 Sam7 nin5 lacUV5-T7 gene I)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
</tr>
<tr>
<td>pGEM-T</td>
<td>3.0kb, Amp’, lacZ</td>
</tr>
<tr>
<td>pT-scFv</td>
<td>3.6kb, insert of the scFv-BRC gene into pGEM-T, Amp’</td>
</tr>
<tr>
<td>pET28a(+)</td>
<td>5.3kb, Kan’, lacI</td>
</tr>
<tr>
<td>pET28a-scFv</td>
<td>6.0kb, insert of the scFv-BRC gene into pET28a(+), Kan’</td>
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clear reactive signals were chosen for sequencing analysis using vector-specific primers of pHEN2 (forward primer; 5'-CAGGAAAACAGCTATGACCATGATT-3', downward primer; 5'-CTATGCGGCCCCATTCAGATC-3'). The nucleotide sequence was verified by automated DNA sequencing (ALFexpressII, Amersham Pharmacia Biotech).

2.4. Construction of Expression Vector and Expression of scFv-BRC

The pT-scFv plasmid was digested with EcoRI and the fragment encoding scFv-BRC was inserted into the pET28a (+) precut by the same restriction enzyme using T4 DNA ligase (pET28a-scFv). The ligation mixture was transformed into E. coli BL21 (DE3). The recombinant E. coli BL21 (DE3) (pET28a-scFv) was grown at 37°C in TY broth (containing 50μg/mL Kan) and induced with IPTG.

2.5. Purification of scFv-BRC and SDS-PAGE

After grown E. coli BL21 (DE3) (pET28a-scFv), the media was centrifuged (4000 rpm for 10 min) and suspended precipitate with 50 mmol/L Tris-HCl (pH 7.2, containing 20 mmol/L NaCl, 20 mmol/L EDTA). After sonication (100 W for 30 min), the supernatant was centrifuged at 12000×g for 20 min and recovered inclusion bodies. For solubilization, the inclusion bodies were suspended in 8 mol/L and incubated at room temperature for 1 hour. After centrifugation (12000×g, 20 min), dialyzed supernatant against 50 mmol/L Tris-HCl (pH 7.2, containing 20 mmol/L NaCl, 20 mmol/L EDTA) was used for refolding.

scFv-BRC was purified through a Ni-NTA column using imidazole gradient [12].

SDS-PAGE was performed on 3.9% (w/v) stacking and 12% (w/v) resolving gels.

2.6. Homology Modeling and Docking of Subtilisin BRC and scFv-BRC

Molecular modeling of the three-dimensional (3D) structures of the scFv-BRC was performed using MODELLER 9.19 modeling software [13] and assessed by PROCHECK [14]. Molecular docking of subtilisin BRC with scFv-BRC was carried out using PATCHDOCK, an online protein-protein docking server (https://bioinfo3d.cs.tau.ac.il/ PatchDock/). This server depends on shape complementarity of soft molecular surfaces to generate the best starting candidate solution [15]. For molecular docking, we used crystal structure of nattokinase from Bacillus subtilis (PDB code 4DWW) as a ligand, because the predicted amino acid sequence of CDS region from subtilisin BRC gene is identical with that of nattokinase (data not shown). The default clustering RMSD 4.0Å was used and the complex type was chosen antibody-antigen. Thereafter, the complementary patches were harmonized to form transformation candidates, which were later refined using FireDock server (http://bioinfo3d.cs.tau.ac.il/FireDock/), an online tool that optimized refined, reshuffled, and rescored the side chains interface of the top 10 candidate solutions. We used the PyMOL software version 2.1.0 for visualization and analyzing the complex interaction.

3. RESULTS AND DISCUSSION

3.1. Selection of Phage Clones Specific to Subtilisin BRC by Phage Display and Sequencing

To obtain specific scFv antibody, the iterative round of panning was performed by using around 10^12 cfu of phages. Upon the completion of every round of panning, the population of the eluted phages was evaluated. Table 2 shows the enrichment of subtilisin BRC-specific scFv antibody fragments achieved through the panning processes. In each panning round, the number of input phages was kept constant at about 10^12 cfu, and the number of output phages increased steadily. After one panning round, the number of output phages was about 1.3×10^7 cfu and finally, it was about 3.7×10^7 cfu after 4 panning rounds. A rising titer of the eluted phages throughout the panning rounds shows that the number and the specificity of the eluted phages were increased consequently.

<table>
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<tr>
<th>Round of Panning</th>
<th>Input Phage/cfu</th>
<th>Output Phage/cfu</th>
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<tbody>
<tr>
<td>1</td>
<td>10^12</td>
<td>1.3×10^6</td>
</tr>
<tr>
<td>2</td>
<td>10^12</td>
<td>1.8×10^7</td>
</tr>
<tr>
<td>3</td>
<td>10^12</td>
<td>2.3×10^8</td>
</tr>
<tr>
<td>4</td>
<td>10^12</td>
<td>3.7×10^7</td>
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</table>

After performing the panning procedure, 50 colonies were randomly selected and analyzed by phage-ELISA to determine their binding capacity.

As shown in Fig. (1), all clones had a positive reaction to subtilisin BRC except clone 39 with different signal intensities. Clone 26 had a much higher signal than the others and this clone was then used for further analysis.

scFv-BRC gene was amplified with colony PCR from E. coli TG1 containing phagemid (pHEN2) selected from the phage antibody library (Griffin.1) and analyzed by agarose gel electrophoresis (data not shown).

For scFv genes cloned into pHEN2 selected Griffin.1, the common primer (forward: 5'-CAGGAAAACAGCTATGACCATGATT-3', downward: 5'-CTATGCGGCCCCATTCAGATC-3') are used. About 635bp of the scFv-BRC gene was obtained and designated as scFv-BRC.

The scFv-BRC gene was sequenced using automated DNA sequencing (Fig. 2).

The size of the scFv-BRC gene is 635bp and it consists of 54bp of heavy chain region (VH), 336bp of light chain region (VL), 45bp of a linker. The size of the structural gene is 435bp. The scFv-BRC gene also contains the same recognition site of restriction enzymes, His-tag, and myc-tag with...
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3.2. Expression of scFv-BRC in E. coli BL21 (DE3) and Purification of scFv-BRC

pT-scFv has two recognition sites of EcoRI and pET28a (+) has one recognition site of EcoRI. pT-scFv plasmid was digested with EcoRI and the 653bp of fragment encoding scFv-BRC was inserted into the pET28a (+) precut by the same restriction enzyme using T4 DNA ligase (pET28a-scFv). The ligation mixture was transformed into E. coli DH5α and revealed insert direction of the scFv-BRC gene. scFv-BRC gene and pET28a (+) have one recognition site of XhoI, the recombinant strain containing scFv-BRC gene inserted forward was selected.

The plasmid was isolated from the selected clone and was transformed into E. coli BL21 (DE3). In order to optimize of the scFv-BRC, we set different additions (0.1, 0.3, 0.5, 0.7, 0.9, 1.1, 1.3mmol/L) of IPTG and different inducing point (0.3, 0.5, 0.7, 0.9, 1.1) of OD_{600} as well as different inducing times (1, 2, 3, 4, 5, 6h) to induce expression. The maximum amount of each experiment was set as 100% and the relative amount of expressed products was calculated by comparisons of those amounts to the maximum amounts. As shown in Fig. (3A), 0.5 mmol/L IPTG was the best choice to induce the expression and decreased in the following higher concentration, because over-accumulating IPTG exhibited toxicity to cells. The amount of scFv-BRC reached the highest value at 0.7 of OD_{600} (Fig. 3B). These results show that IPTG inducer might be added at the middle or terminal stage of the exponential growth phase. The highest amount was obtained after being induced for 5 hours (Fig. 3C).

The selected recombinant E. coli BL21 (DE3) (pET28a-scFv) was inoculated into TY broth and grown at 37°C with shaking (150rpm). When OD_{600} reached 0.7, IPTG (final concentration 0.5 mmol/L) was added and growth was continued at 25°C for 5 hours.

scFv-BRC was not expressed without IPTG induction. This result shows that scFv-BRC gene was recombined with pET28a (+) correctly and expressed in E. coli BL21 (DE3). After the cells were grown, the cells were recovered by centrifugation and sonicated. The scFv-BRC was detected only in the precipitate, and this shows that scFv-BRC was accumulated in inclusion bodies.

scFv gene cloned into pHEN2 has His tags and scFv can purify with immobilized metal affinity chromatography (IMAC) using Ni-NTA column. After solubilization and refolding of expressed scFv-BRC, it is purified through a Ni-NTA column using imidazole gradient (0.1–0.13 mol/L). According to SDS-PAGE analysis, a strong band with about 26kDa molecular weight was detected. The amount of expressed scFv-BRC was about 50 mg/L (about 40% of the total protein of cells).
3.3. Homology Modeling of scFv-BRC

The 3D structure model of scFv-BRC was constructed by MODELLER 9.19, and MODELLER 9.19 generated a pool of theoretical structure models for the scFv-BRC. The crystal structure of anti-H4K20me1_scFv (PDB code 5B3N) was used as the template for scFv-BRC homology modeling [16]. The best structure model was selected from the pool by the value of the DOPE assessment score (Fig. 4A). The quality of the model structure was evaluated using a Ramachandran plot obtained from PROCHECK. As shown in Fig. 4B, about 89.8% amino acid residues are present in the most favored regions and only Ala105 is present in the disallowed regions. These results showed that the scFv-BRC model was suitable for molecular docking. scFv-BRC was composed of antiparallel-folded β-sheets connected by loops (Fig. 4A), displaying the characteristic variable regions of immunoglobulin folding [18].
Fig. (3). Effects of IPTG concentration (A) inducing point (B) time (C) to the expression of scFv-BRC in *E. coli* BL21 (DE3) (pET28a-scFv).

Fig. (4). Three-dimensional homology model of scFv-BRC. (A) 3D model of scFv-BRC. CDRs are depicted as a stick model. (B) Ramachandran plot of scFv-BRC.
3.4. Docking of scFv-BRC with Subtilisin BRC

To visually evaluate the interaction of the subtilisin BRC with scFv-BRC, the subtilisin BRC molecules were docked onto scFv-BRC using PATCHDOCK and FIREDOCK server. As mentioned in the MATERIALS AND METHODS section, the crystal structure of nattokinase from Bacillus subtilis natto (PDB code 4DWW) was used as a ligand. The models with the lowest energy docking were selected from the resulting docking models. According to the docking results, the global energy of the complex was estimated to be -56.29kJ/mol. The final configuration of the docking complex of subtilisin BRC and scFv-BRC is shown in Fig. (5).

Furthermore, the results showed that amino acid residues in subtilisin BRC for binding with scFv-BRC are Tyr6, Ser182, Ser204 and Gln206 (Fig. 5A). In comparison with the main binding site of nattokinase for its substrates, we think that these amino acid residues are not relevant to that [19].

CONCLUSION

In this study, we have selected the single-chain variable fragment antibody (scFv) against subtilisin BRC using phage display and evaluated the interaction of the scFv-BRC with subtilisin BRC using molecular docking.

Phage display technology has many advantages in the selection of antibodies including rapidity, ease, robust and well-established E. coli, specificity and short panning round [17]. In the present study, this technology was applied to select scFv against the subtilisin BRC from Griffin.1 library.

The scFv against subtilisin BRC selected using phage display showed relatively strong binding energy with subtilisin BRC. The amino acid residues in subtilisin BRC for binding with scFv-BRC were not relevant to that in subtilisin BRC for binding with its substrates. These results suggested that the scFv-BRC can be used as a ligand for detection and affinity purification of subtilisin BRC.

Further research is expected on binding characteristics between subtilisin BRC and scFv-BRC and detection of subtilisin BRC using scFv-BRC as the affinity ligand.

LIST OF ABBREVIATIONS
CDR = Complementarity Determining Region
scFv = Single Chain Variable Fragment Antibody

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
Not applicable.

HUMAN AND ANIMAL RIGHTS
No Animals/Humans were used for studies that are basis of this research.

CONSENT FOR PUBLICATION
Not applicable.

FUNDING
None.

CONFLICT OF INTEREST
The authors declare no conflict of interest, financial or otherwise.

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REFERENCES
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