강 창 수 교수 지도

석사학위 청구논문

# Isolation of CD200 specific peptides based on phage display technology

-Discovery of peptide sequences for immune checkpoint regulation-

2024

성신여자대학교 대학원

생물학과

석 수 아

# Isolation of CD200 specific peptides based on phage display technology

-Discovery of peptide sequences
for immune checkpoint regulation-

A Master's Thesis
Submitted to the
Graduate School of Sungshin Women's University

in partial fulfillment of the requirements
for the degree of
Master of Biology

Sua Seok

November, 2023

This is to certify that we have examined the

Master's Thesis of

Sua Seok

Submitted to Department of Biology

Approved as to style and content:

Thesis Advisor .....

Committee Chairman .....

Committee Member .....

Committee Member .....

The Graduate School of

Sungshin Women's University

# ABSTRACT

## Isolation of CD200 specific peptides based on phage display technology

Sua Seok
Department of Biology
Graduate School of
Sungshin Women's universiity

Immune checkpoint inhibitors represent a class of drugs designed to impede the activity of proteins constituting the immune checkpoint expressed on T cells or tumor cells. These inhibitors play a crucial role in assisting T cells to eliminate cancer cells, providing a potential avenue for anticancer therapy. CD200 (OX-2 membrane glycoprotein) can be considered an interesting target for immune checkpoints. The interaction between CD200 and its receptor, CD200R, has been implicated in immune-related diseases, including allergic diseases, infections, arthritis, transplantation, and autoimmune diseases such as multiple sclerosis and systemic lupus erythematosus. Experiments were designed to screen for anti-CD200 peptides that bind to CD200 to interfere with the binding of CD200 to CD200R. To obtain CD200, the protein was expressed using a mammalian expression system, with HEK (Human embryonic kidney) cells chosen for their high-efficiency expression. Ni-NTA chromatography was performed for protein purification, followed by protein MS to check for impurities. Phage display technology, a screening method recognizing interesting

candidates based on binding affinity for a given target molecule, was used to screen peptides binding specifically to CD200. After three rounds of biopanning, phage plaques were selected, amplified, purified, and DNA sequencing was performed. As a result, the 12-mer peptide sequence was identified. The sequence chosen through further analysis is anticipated to possess the potential as an antagonist capable of blocking the interaction between CD200 and CD200R.

# CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# I. INTRODUCTION

Immune checkpoint inhibitors are pharmaceuticals designed to inhibit the activity of proteins that constitute the immune checkpoint expressed on T cells or tumor cells. These inhibitors play a pivotal role in assisting T cells in eradicating cancer cells and hold potential for use in anticancer therapy [1]. CD200, a 45 kDa transmembrane immunoregulatory protein known as OX-2 membrane glycoprotein, belongs to the immunoglobulin superfamily [2]. CD200 is expressed on various cell types, including thymocytes, activated T cells, B cells, dendritic cells (DC), vascular endothelial cells, hair follicle cells, central nervous system, and retina [3]. The interaction between the CD200 and its receptor CD200R has been implicated in immune-related diseases such as allergic diseases, infections, arthritis, transplantation, and autoimmune diseases like multiple sclerosis and systemic lupus erythematosus [3]. Therefore, CD200 may be an intriguing target for immune checkpoint inhibition. Both CD200 and CD200R are type 1 membrane proteins, penetrating the membrane only once, which facilitates interaction in the extracellular region. (Figure 1.)

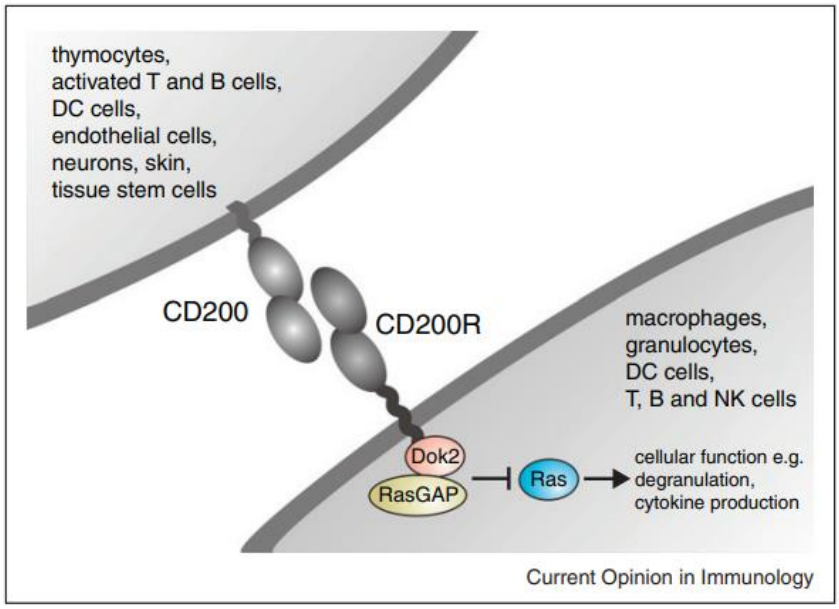



**Figure 1. Expression pattern and interactions of CD200 and CD200R.** (Rygiel *et al.*, 2012) CD200 and CD200R are both type 1 transmembrane proteins, with their extracellular regions engaging in binding and interaction. CD200 is expressed in various cell types, including thymocytes, activated T and B cells, dendritic cells (DC), endothelial cells, neurons, skin, and tissue stem cells. In contrast, CD200R is expressed in macrophages, granulocytes, DC cells, as well as T, B, and NK cells.

Table 1. Information of Human CD200 Isoform 1.

| Human CD200 (OX-2 membrane glycoprotein Isoform 1) | |
|---|---|
| Accession # | P41217-1 |
| Length | 278 a.a |
| Mass (Da) | 31,264 Da |
| Signal peptide | 1~28 |
| Extracellular region | 31~232 |
| Transmembrane region | 233~259 |
| Cytoplasmic region | 260~278 |
| Glycosylation site | 95, 103, 110, 157, 181, 190 |
| Calculated Molecular Mass (Extracellular region) | 23.8 kDa |
| Observed Molecular Mass (SDS-PAGE) | 38-54 kDa, Protein migrates due to glycosylation |

Recombinant proteins produced through mammalian expression systems exhibit excellent quality and efficacy, explaining why a significant number of protein drugs are manufactured in animal cells [4]. The notable advantage of mammalian cell expression is that the mammalian cells can appropriately and efficiently recognize signals for the synthesis, processing, and secretion of eukaryotic proteins [5]. Consequently, expression systems employing mammalian cells for recombinant protein production can ensure proper protein folding, post-translational modifications, and protein assembly, all crucial for achieving complete biological activity [6]. Various mammalian cell lines have been employed for protein expression, with the most common choices being HEK293 (Human embryonic kidney) and CHO (Chinese hamster ovary) cells [5]. Notably, HEK293 cells possess the advantage of producing proteins most similar to those naturally synthesized by humans.

In the protein expression process, transformation methods encompass transient expression and stable expression. Transient expression involves the transient, small-scale production of antibodies in a mammalian host, whereas stable expression refers to a stable, nearly permanent, large-scale production of recombinant proteins [7]. Unlike

stable expression, transient expression is a method capable of producing high-purity proteins inexpensively and quickly [8]. Transient transfection is commonly employed when rapid confirmation is essential during the initial selection process [7,9].

The phage display system is a valuable tool for easily selecting peptides that specifically bind to target proteins. This selection is achieved through the display of peptides on the minor coat protein (GIII protein) of the single-stranded filamentous bacteriophage M13 [10]. (Figure 2.) The M13 bacteriophage forms particles with single-stranded circular DNA through budding from the bacterial cell wall. The process of M13 infection initiates with adsorption, where the N2 domain of the G3P coat protein binds to the ends of F pili on the Escherichia coli host surface [10]. (Figure 3.)

M13KE is a simple M13 derivative in which cloning sites have been introduced at the 5′ end of gene III for display of short peptide sequences as N-terminal pIII fusions [11]. (Figure 4.) As it is a phage, not a phagemid vector, each virion's surface features all five copies of pIII fused to the cloned peptide.

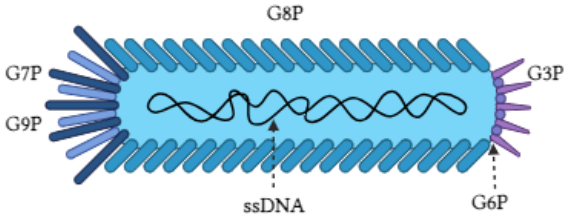



Figure 2. M13 Bacteriophage.

This filamentous phage carries a single-stranded DNA (ssDNA).

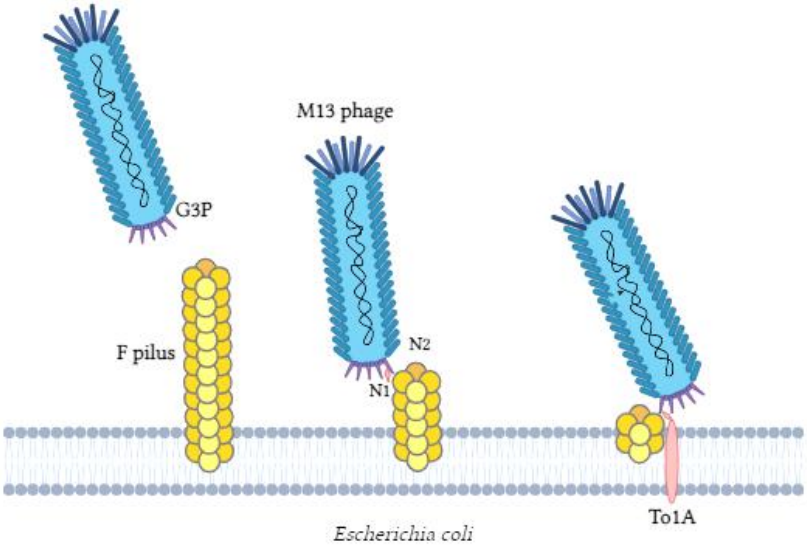



Figure 3. Infection of *E.coli* by the M13 phage.

The G3P phage protein binds to the tip of the F pilus on *E.coli*.

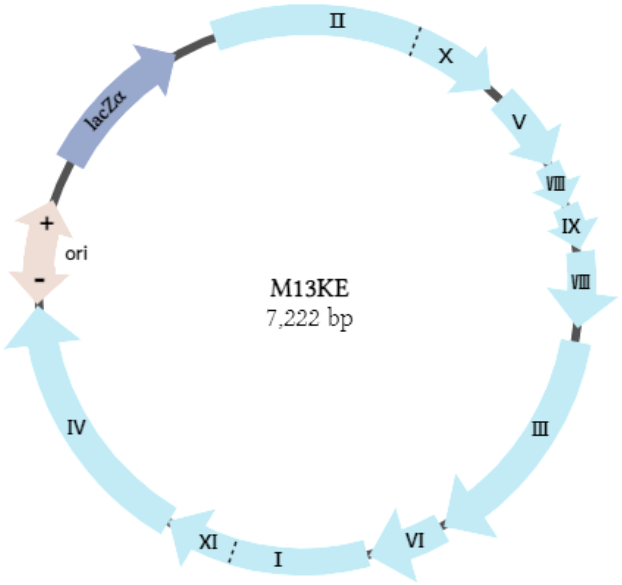



Figure 4. M13KE vector map.

M13KE is a derivative of M13mp19, designed for expressing peptides as N-terminal pIII fusions in phage display applications. The M13KE vector incorporates the lacZ$\alpha$ insert positioned in the intergenic region between genes IV and II, effectively interrupting the replication enhancer of the (+) strand origin. [11]

# II. MATERIALS AND METHODS

## 1. Cell lines, plasmids, and peptide libraries used in this study

Table 2 presents the bacterial strains, plasmids, mammalian cell lines, and peptide libraries utilized in this study.

## 2. Design of recombinant DNA

In order to express proteins in HEK cells utilizing a mammalian expression system, the pcDNA3.4 vector, recognized as a mammalian expression vector, was employed [12]. (Figure 5.) The CD200 sequence, representing the extracellular region of the canonical human CD200 Isoform 1, was selected. To ensure high-efficiency expression, the chosen CD200 sequence was specifically optimized for the human species [13]. Given that CD200 is a type 1 membrane protein that penetrates the membrane only once, gene cloning focused solely on the extracellular region where CD200-CD200R interaction occurs

[14]. For protein expression in animal cells, the recombinant sequence included the start codon, Kozak sequence, secretion signal, and stop codon, with the addition of a histidine tag for purification purposes [15]. (Table 3.). As HEK293F cells are suspension culture cells, an N-terminal secretion signal was incorporated to ensure protein secretion into the media after transfection. To purify the expressed protein using Nickel affinity chromatography, a 10-his tag was attached to the C-terminal. Sequencing was performed to confirm that the CMV promoter, insert, and other sequences of the recombinant plasmid were cloned without mutation [16]. All sequencing processes in this study were performed through an online sequencing service based on Sanger sequencing provided by Macrogen. To obtain the necessary amount of DNA for transfection, the pcDNA3.4 with the CD200 plasmid was extracted and purified using the AccuPrep® Nano-Plus Plasmid Midi Extraction kit (Bioneer). The purity of the extracted plasmid was assessed using agarose gel electrophoresis.

Table 2. Information on Bacterial strains, plasmid, cell line and peptide libraries used in this study.

| Genotypes and features | |
|---|---|
| *E.coli* | |
| DH5a | *fhuA2⊿(argF-lacZ)U169 phoA glnV44 Ø80⊿(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17* |
| K12 ER2738 | *F ´ proA+B+ lacIq ⊿(lacZ)M15 zzf::Tn10(TetR)/fhuA2 glnV ⊿(lac-proAB) thi-1 ⊿(hsdS-mcrB)5* |
| Expression vector | |
| pcDNA3.4 | CMV promoter, pUC Origin, bla |
| Cell line | |
| Expi293F™ cell (Gibco™) | Human embryonic kidney cell, suspension culture cell, higher protein expression levels, more rapid cell growth and higher culture viabilities than other 293 cells |
| Peptide library | Infectious M13 particles displaying random peptide library on pⅢ coat protein, 1 x $10^{13}$ pfu/ml |
| Sequencing primer | |
| -96glll | 5'-CCC TCA TAG TTA GCG TAA CG-3' |

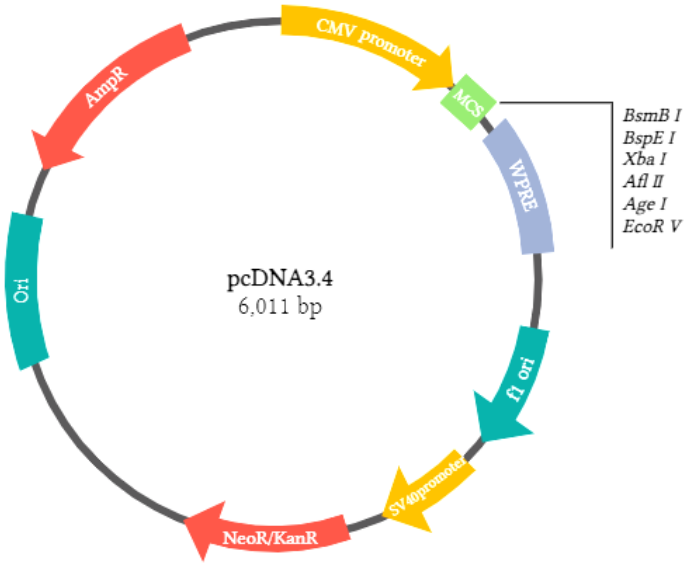



Figure 5. pcDNA3.4 vector map.

The pcDNA3.4 vector incorporates the following elements:

-WPRE downstream of the cloning site to enhance transcript expression

-pUC origin for high copy replication and maintenance of the plasmid in *E.coli*

-Full-length human CMV immediate-early promoter

-Ampicillin (bla) resistance gene for selection in *E.coli*

Table 3. Designing of recombinant CD200.

| | |
|---|---|
| Kozak sequence | GCCACC |
| Secretion signal | |
| Amino acid sequence | METDTLLLWVFLLLWVPGSTG |
| Optimized sequence | ATGGAAACCGACACCCTGCTGCTG TGGGTGCTGCTGCTTTGGGTCCCC GGCAGCACCGGC |
| Histidine tag | |
| Amino acid sequence | HHHHHHHHHH |
| Optimized sequence | CACCACCACCACCACCACCATCAT CACCAC |
| Stop codon | TGA |

## 3. Cell culture and Transient expression

Transient expression was employed to expedite protein expression, saving time and reducing costs for assay development and screening [17]. Expi293F™ Expression System Kit (Gibco™), designed for rapid and high-yield protein production in mammalian cells using transient expression technology, was utilized for cell culture and protein expression. The HEK293 cell line, known for its ease of culture and high transfection efficiency, was used, and Expi293F™ Cells, derived from the HEK293 cell line, were employed [18]. These cells are maintained in suspension culture and optimized for high-density growth in Expi293F™ expression medium. Notably, Expi293F™ Cells exhibit high transfectability, leading to superior transient protein yields compared to standard 293 cell lines [19].

For cell culture, 30 ml of pre-warmed Expi293 Expression Medium was added to a 125 ml polycarbonate, disposable, sterile, vented Erlenmeyer shaker flask. Thawed cells were transferred to the flask and incubated with shaking in a 37℃ incubator with ≥80% relative humidity and 8% $CO_2$. Upon reaching a cell density of 3-5 x $10^6$ viable cells/ml, it was diluted to a final density of 3 x $10^6$ viable cells/ml and allowed to grow overnight. Transfection was performed when the cell

density reached 4.5−5.5 x $10^6$ cells/ml, with cell viability over 95%. For transfection, 1 $\mu$g per mL of culture volume of plasmid DNA was added to the cell culture. The incubation continued in a 37℃ incubator with a humidified atmosphere of 8% $CO_2$ in the air on an orbital shaker. On the day after transfection, two enhancers were added to support high−density transient transfection and improve protein production. Cell viability was measured daily, and cells were harvested when it dropped to approximately 70%. Negative and positive control experiments were conducted alongside [20,21]. The background band observed during HEK cell culture was noted through a negative control that excluded the DNA transfection process. The expression of the CD200 was confirmed by comparing this with the band that appeared after CD200 transfection [20]. Additionally, positive controls were tested to ensure the study was conducted correctly and produced appropriate results [21]. To confirm transfection and expression in Expi293F™ cells, the antibody expression positive control vector provided in the Expi293F™ Expression System Kit (Gibco™) was used. At this time, the pcDNA3 .4 plasmid expressing the heavy and light chains of rabbit IgG served as the positive control vector, with a heavy:light chain ratio of 1:2.

## 4. Protein Purification

Both CD200 protein and IgG antibody, expressed as a positive control, underwent purification using distinct chromatography methods. Initially, IgG, the positive control, was purified utilizing protein A chromatography [22]. The MagListo™ Protein A Kit (Bioneer) facilitated antibody purification through magnetic separation using Protein A Magnetic Nanobeads [23]. The magnetic nanobeads were equilibrated with Binding & Washing buffer. Following the loading of the IgG culture supernatant sample onto the nanobeads, incubation in a rotator for 1 hour at room temperature allowed the protein to bind to the beads. Subsequent washes with Binding & Washing buffer were performed twice. Elution was achieved by incubating for 1 minute at room temperature with elution buffer, and 10% neutralization buffer was added to the eluate.

Subsequently, nickel affinity chromatography was employed for the purification of the histidine tagged CD200 [24]. This process utilized Ni-NTA resin, which binds nickel ions to NTA (Nitrilotriacetic acid), a material capable of chelating metal ions [25]. Nuvia™ IMAC Ni-Charge Resin (BIO-RAD) was the chosen resin. The poly-histidine

tag of the recombinant protein bound to the metal ion of the resin with micromolar affinity [26]. The resin was washed to eliminate proteins that did not specifically interact with nickel ions. Two washes were conducted using a buffer containing 5 mM and 10 mM of imidazole. Subsequently, elution was performed with an elution buffer containing an increased imidazole concentration. To determine the optimal imidazole concentration, elution buffers of 30 mM, 100 mM, 150 mM, 200 mM, and 250 mM were employed, with each resulting in a distinct elution volume [27]. Ultimately, the elution buffer containing 250 mM imidazole, demonstrating the highest concentration, was selected.

## 5. SDS-PAGE and Western Blot analysis

### 1) SDS-PAGE Procedure

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is a protein separation technique that separates proteins based on their size [28]. The purified protein was checked for expression and size using the SDS-PAGE. An SDS-PAGE gel consisting of a 10% resolving gel and a 5% stacking gel was prepared [29]. Each protein sample was mixed with 4X sample buffer containing DTT, boiling at 95℃ for 5 minutes [30], and then 10 ㎕ of boiled mixture was loaded per well and run with a constant current of 30 mA. The composition of buffers used for SDS-PAGE and WB (Western Blot) are shown in Table 4.

### 2) SDS-PAGE Visualization

Following SDS-PAGE, initially invisible electrophoresed samples required a visualization. The expression and size of CD200 were detected through Coomassie Brilliant Blue staining, with Western Blot confirmation using antibody treatment [31]. First, Coomassie Brilliant Blue R-250 staining solution was used as a visualization method by

dyeing the gel [32]. The gel was stained with Coomassie Brilliant Blue staining solution by incubating it for 1 hour on a rocker. After staining, the gel was transferred to the destaining solution, incubated for an additional 2 hours, and then subjected to chemiluminescence detection using Chemi-doc (BIO-RAD).

### 3) Western Blot Procedure

For Western Blot, protein transfer from the gel to the polyvinylidene fluoride (PVDF) membrane was achieved by passing an electric current [33]. Immobilon®-P PVDF Membrane (Millipore®) was used as a membrane. The transfer used Trans-blot® turbo™ (BIO-RAD), creating a gel-membrane sandwich uploaded to the cassette. The membrane, placed on the bottom cassette side (positive charge), and the gel on the top cassette side (negative charge), with filter paper on both sides, ran for 7 minutes, 1.3A constant, up to 25V—an appropriate setting for CD200's molecular weight. After the transfer completion, all bands on the gel were transferred to the membrane. This membrane underwent a 1 hour incubation in blocking buffer (3% BSA dissolved in 0.1% TBS-T) at half-power on a rocker. Blocking prevented nonspecific antibody binding [34]. Subsequently, the membrane was

treated with antibodies, using a simplified, one-step process utilized peroxidase-conjugated antibody binding to his-tag. Anti-polyHistidine-Peroxidase antibody, Mouse monoclonal (Sigma-Aldrich®) was utilized. Antibody treatment was also performed under the same conditions as blocking, and then washed three times for 10 minutes with 0.1% TBS-T.

4) Western-Blot Visualization

The membrane was then treated with an enhanced chemiluminescence (ECL) substrate solution that produces light when exposed to HRP [35] and incubated for 5 minutes using an EZ-Western Lumi Pico Alpha kit (DoGenBio). The reacted membrane was detected and imaged with Chemi-doc (BIO-RAD).

Table 4. SDS-PAGE and Western Blot buffer solution components

| Buffer | Components |
|---|---|
| 5X Running buffer | 0.125 M Tris-base, 0.96 M Glycine, 0.5% SDS, Dilute to 1 X before use |
| Methanol:Acetic acid solution | 50% Methanol, 10% Glacial acetic acid |
| Coomassie Brilliant Blue staining solution | Dissolve 0.25 g of Coomassie Brilliant blue R-250 per 100 ml of Methanol:Acetic acid solution |
| Destaining solution | 30% Methanol, 10% Glacial acetic acid |
| 10X Transfer buffer | 0.25 M Tris-base, 1.92 M Glycine (pH 8.3) Dilute to 1X before use (add 20% methanol) |
| Blocking buffer | 3% BSA dissolved in 0.1% TBS-T |

## 6. Bradford assay

Bradford assay was conducted to determine the concentration of dissolved protein. Coomassie Brilliant Blue G-250 was employed to measure protein concentration by causing a shift in the maximum absorption of the dye to 595 nm [36]. The Bio-Rad Protein Assay Dye Reagent Concentrate (BIO-RAD) served as the dye solution, prepared by diluting 1 part Dye Reagent Concentrate with 4 parts distilled water (DW). Additionally, bovine serum albumin (BSA) was dissolved to create five levels of dilution for protein standards, ranging from 0.05 mg/ml to 0.5 mg/ml. Subsequently, 10 ㎕ of both standard and CD200 sample solutions were added to separate microtiter plate wells. Then, 200 ㎕ of diluted dye reagent was added to each well and thoroughly mixed. The mixed samples were incubated at room temperature for at least 5 minutes, followed by absorbance measurement at 595 nm using the SpectraMax® M Series Multi-Mode Microplate Readers (Molecular Devices) A standard curve was created using the absorbance values of the standard samples [37], and protein concentrations were calculated by substituting the protein sample absorbance values into the calibration curve equation.

## 7. Concentration of proteins

Protein concentration was conducted to ensure an adequate amount of protein for the subsequent step, biopanning. The Amicon® Ultra-15 Centrifugal Filter Unit (Millipore) was employed for concentration [38], specifically utilizing a 3K (3,000 MWCO - Molecular Weight CufOff) device from the product family. 15 ml of CD200 media sample was added to the Amicon® Ultra filter device and centrifuged at 4000 g for 1 hour at room temperature, using a swinging-bucket rotor centrifuge. Following centrifugation, the concentrate volume was approximately 200 μl. DW was added to reach a final volume of 1.5 ml, resulting in a 10-fold concentrated media. Subsequently, Ni-NTA chromatography was performed once again using the concentrated medium, ultimately yielding a 10-fold concentrated protein eluate.

## 8. Protein Mass Spectrometry

Secondary purifications were performed to assess protein impurity, employing size exclusion chromatography (SEC), reverse-phase chromatography (RP), and Intact Mass Spectrometry (Intact MS). These experiments were carried out in Professor Byungjun Ko's laboratory.

### 1) Size Exclusion Chromatography (SEC)

SEC analysis, performed first, aimed to identify potential protein aggregation [39]. This technique separates proteins based on size, with larger proteins eluting faster due to exclusion from most pores. A single monomeric peak in the SEC analysis result indicates the absence of protein aggregation.

### 2) Reverse-Phase Chromatography (RP-HPLC)

Reverse-phase high-performance liquid chromatography (RP-HPLC) was employed to measure protein impurities [40]. RP-HPLC separates analytes based on their hydrophobicity, with proteins of high purity appearing as sharp, single peaks. Gradient elution was achieved

using a mobile phase consisting of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in ACN).

### 3) Intact Mass Analysis

Following treatment with PNGase F to remove N-linked glycans, the molecular weight was determined through Intact mass analysis [41]. PNGase F, an enzyme that hydrolyzes and removes N-linked oligosaccharides, facilitates accurate confirmation of the protein's accurate molecular weight. The experimental conditions for each analysis are detailed in Table 5.

Table 5. Conditions for analysis of protein mass spectrometry.

a. SEC (Size-Exclusion Chromatography)

| Device | Manufacturer |
|---|---|
| Waters Alliance e2695 Separations Module | Waters™ |
| Waters 2998 Photodiode Array (PDA) Detector | Waters™ |
| TSKgel® G3000SWXL HPLC Column | TOSOH |

| Parameter | Condition |
|---|---|
| Flow rate | 1 ml/min |
| Column temperature | 25℃ |
| Sample temperature | 4℃ |
| Detection | UV 280 nm |
| Needle wash | 50% ACN |
| Seal wash | 10% ACN |
| Run time | 20 min |
| Gradient | Isocratic (1X PBS, pH 7.4) |

b. RP (Reverse-Phase Chromatography)

| Device | Manufacturer |
|---|---|
| Waters Acquity UPLC Sample Manager | Waters™ |
| Waters Acquity UPLC Binary Solvent Manager | Waters™ |
| BioResolve RP mAb Polyphenyl Column | Waters™ |

| Parameter | Condition |
|---|---|
| Flow rate | 0.5 ml/min |
| Column temperature | 80℃ |
| Sample temperature | 4℃ |
| Detection | UV 280 nm |
| Needle wash | 100% DW |
| Seal wash | 10% ACN |
| Run time | 15 min |
| Gradient | A: 0.1% TFA in DW<br>B: 0.1% TFA in ACN |

c. Intact Mass

| Device | Manufacturer |
|---|---|
| TUV Detector | Waters™ |
| Sample Manager FTN-1 | Waters™ |
| Binary Solvent Manager | Waters™ |
| Xevo G2-XS QTof | Waters™ |
| BioResolve RP mAb Polyphenyl Column | Waters™ |

| Parameter | Condition |
|---|---|
| Column temperature | 80℃ |
| Sample temperature | 4℃ |
| Needle wash | 80% ACN |
| Seal wash | 10% ACN |
| Run time | 6.5 min |

Gradient

| Time | Flow (ml/min) | %A | %B | Curve |
|---|---|---|---|---|
| Initial | 0.400 | 95.0 | 5.0 | Initial |
| 1.00 | 0.400 | 95.0 | 5.0 | 6 |
| 1.01 | 0.200 | 95.0 | 5.0 | 6 |
| 3.50 | 0.200 | 5.0 | 95.0 | 6 |
| 3.70 | 0.400 | 5.0 | 95.0 | 6 |
| 4.00 | 0.400 | 95.0 | 5.0 | 6 |
| 4.50 | 0.400 | 5.0 | 95.0 | 6 |
| 5.00 | 0.400 | 95.0 | 5.0 | 6 |
| 5.50 | 0.400 | 95.0 | 5.0 | 6 |

## 9. Phage display

The phage display process was executed using Ph.D Phage Display Peptide Library kit v2 (New England Biolabs).

### 1) Surface Panning (Direct target coating)

Recombinant human CD200, prepared at 100 $\mu$g/ml in coating butter (0.1M $NaHCO_3$, pH8.6), was added to microtiter wells and incubated overnight at 4℃ to coat the plate with the target. Following blocking with blocking buffer (5 mg/ml BSA in 0.1 M $NaHCO_3$, pH 8.6) for 1 hour at 4℃, the plate was washed with 0.1% TBS-T (Tris-Buffered Saline + 0.1% [v/v] Tween-20). The phage library, diluted to 2 x $10^{11}$ pfu/ml in TBS-T, was added to the coated plate and incubated at room temperature for 1 hour. Nonbinding phages were removed through washing with 0.1% TBS-T, and bound phages were eluted by adding elution buffer (0.1 M sodium acetate, pH 3.0) and incubating for 30 minutes at room temperature. The obtained phage elution was adjusted to neutral pH by adding 1/5 the neutralization buffer (1.5 M Tris-Cl, pH 8.8). The elution was then amplified by shaking incubation at 37℃ for 4.5 hours in ER2738 culture in early-

log phase. After amplification, the supernatant was obtained by centrifugation for 10 minutes, and 1/6 volume of 20% PEG/2.5 M NaCl was added. Phages were allowed to precipitate overnight at 4℃. Centrifugation was performed at 12000 g for 15 minutes, the supernatant was removed, and the pellet was dissolved with TBS. 1/6 volume of 20% PEG/2.5 M NaCl was added and placed on ice for 1 hour to allow reprecipitation. The supernatant was removed by centrifugation at 14,000 rpm for 10 minutes at 4℃, and the pellet was dissolved in TBS and finally an amplified eluate was obtained. This entire process constituted one round and was repeated for a total of 3 rounds.

2) Titration

The phage elution was prepared by serial dilution, and the ER2738 culture was incubated in LB broth to mid-log phase (OD600 ~ 0.5). For infection, 200 $\mu$l of ER2738 culture and 10 $\mu$l of serially diluted phage were mixed. Infected cells were transferred to 3 ml of Top agar warmed to 45℃ and poured onto an LB/IPTG/X-gal plate. After overnight incubation at 37℃, the titer was measured by counting blue plaques.

3) Plaque Amplification for Sequencing

Blue plaques from the third round of panning were picked and added to ER2738 culture grown to the early-log phase in LB broth. The mixture was incubated with shaking at 37℃ for 6 hours. The supernatant, an amplified phage stock, was obtained by centrifugation at 8000 rpm for 20 minutes.

4) Phage DNA Isolation for Sequencing

For sequencing, 1/6 volume of 20% PEG/2.5 M NaCl was added to the amplified phage stock and left on ice for 1 hour to precipitate the phage. After centrifugation at 8000 rpm for 20 minutes at 4℃, Tris-EDTA (TE) buffer was added to the pellet to suspend it. Phenol and chloroform were added, centrifuged, and the supernatant was transferred. This process was repeated until no white residue was formed at the interface. After adding 1/10 the amount of 3 M sodium acetate and 2 to 2.5 times the amount of ethanol, it was placed at -20℃ to allow DNA precipitation. After centrifugation at 4℃ and washing with 70% ethanol, the pellet dried in vacuum was dissolved in 20 $\mu$l of 10 mM Tris, and single strand DNA of phage was obtained.

# III. RESULTS

## 1. Recombinant DNA Cloning

CD200, the protein to be expressed, was inserted into the pcDNA3.4 vector, a mammalian expression vector. At this time, the cloning site was selected so as not to damage the CMV promoter sequence of the vector, and it was also inserted between two other restriction enzymes sites (Xba I , Age I ) to check whether the cloning was successful. pcDNA3.4 w/CD200 plasmid was analyzed by 0.8% agarose gel electrophoresis. (Figure 6.) A band of the correct size was observed in both the plasmid preparation results and the digestion results at the two-restriction enzyme sites and was confirmed once again through sequencing analysis. (Figure 7.) It was confirmed that the Kozak sequence and secretion signal, which are the sequences required for the HEK293 expression system, were successfully inserted, and the CMV for primer binding site, histidine tag, and CD200 sequence also appeared without mutation. Therefore, it was determined that cloning was successful.

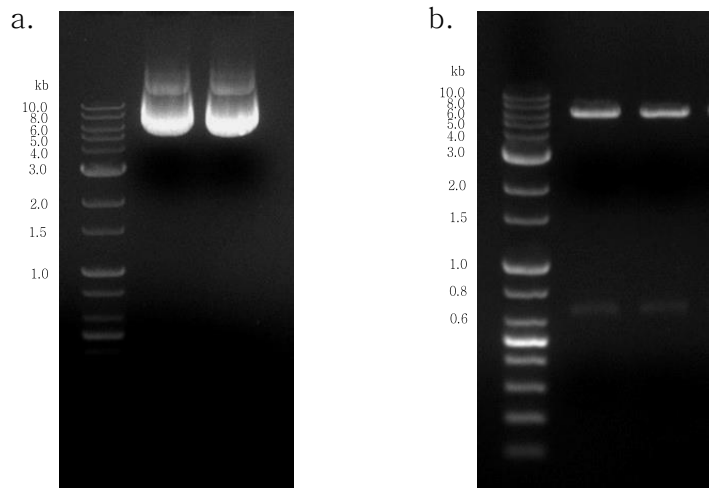



**Figure 6: 0.8% agarose gel electrophoresis verification of cloned recombinant pcDNA3.4 w/CD200 plasmid.**

a. Extracted plasmid DNA from midi-prep. b. Restriction enzyme double cut (Xba I, Age I) to confirm CD200 insert. Following enzymatic treatment, the plasmid exhibited a total length of 6686 bp, with a DNA insert size of 711 bp and a vector size of 5975 bp.

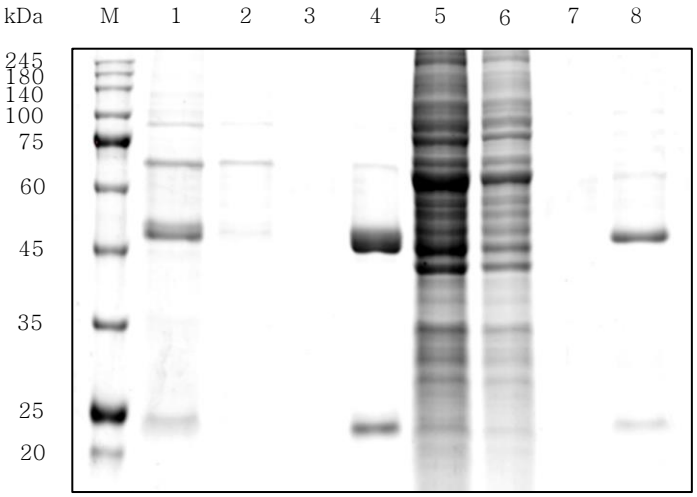

Figure 7. Sequencing analysis results of pcDNA3.4 w/CD200. a. CMV for primer binding site. b. Kozak sequence. c. Secretion signal. d. optimized CD200 sequence. e. histidine-tag.

## 2. CD200 Expression and Purification

Following the expression of proteins in HEK cells via suspension culture, cell harvesting occurred at roughly 70% viability. A positive control was used to confirm that the experimental procedures such as cell culture and transfection were performed correctly. The positive control used pcDNA3.4 w/IgG plasmid, and all other processes were performed the same. IgG purification, using protein A chromatography −well−suited for IgG antibodies− confirmed the expression of both the 50 kDa heavy chain and the 25 kDa light chain. (Figure 8.) Additionally, protein expression was validated by comparison with a negative control group that underwent parallel experimentation, excluding the DNA transfection process. (Figure 9.) The supernatant from the harvested cell culture, anticipated to contain proteins, underwent purification using nickel affinity chromatography. To find the most efficient elution concentration, various elution concentrations (30, 100, 150, 200, and 250 mM imidazole) were tested, with the 250 mM imidazole solution exhibiting the highest efficiency selected as the final elution buffer. (Figure 10.) To confirm protein secretion during expression, the pellet was lysed and purified similarly to the

supernatant, with minimal protein detection in the lysate elution, affirming successful protein secretion (Figure 11.)

Western blot analysis was conducted to verify that the band observed in Coomassie Brilliant Blue staining represented the desired recombinant protein. (Figure 12.) Given the presence of a his-tag in the recombinant CD200, treatment with an anti-histidine-HRP conjugated antibody was performed. This confirmed the presence of the desired protein in both the cell culture supernatant and the purified protein elution.




Lane M : Size marker

Lane 1 : IgG culture supernatant

Lane 2 : IgG media unbound fraction

Lane 3 : IgG media washing fraction

Lane 4 : IgG media elution

Lane 5 : IgG culture lysate

Lane 6 : IgG lysate unbound fraction

Lane 7 : IgG lysate washing fraction

Lane 8 : IgG lysate elution

**Figure 8. IgG purification results using Coomassie Brilliant Blue staining as a positive control.** 10 $\mu$l of sample was applied to each lane. Lanes 1−4 depict purification results of the culture supernatant, while lanes 5−8 show results for the lysed pellet. Unbound sample assessment is in Lanes 2 and 6, and successful washing is confirmed in Lanes 3 and 7. Expression of the 50 kDa heavy chain and the 25 kDa light chain is evident in eluates from lanes 4 and 7. The darker band in lane 4 indicates efficient secretion during protein expression.

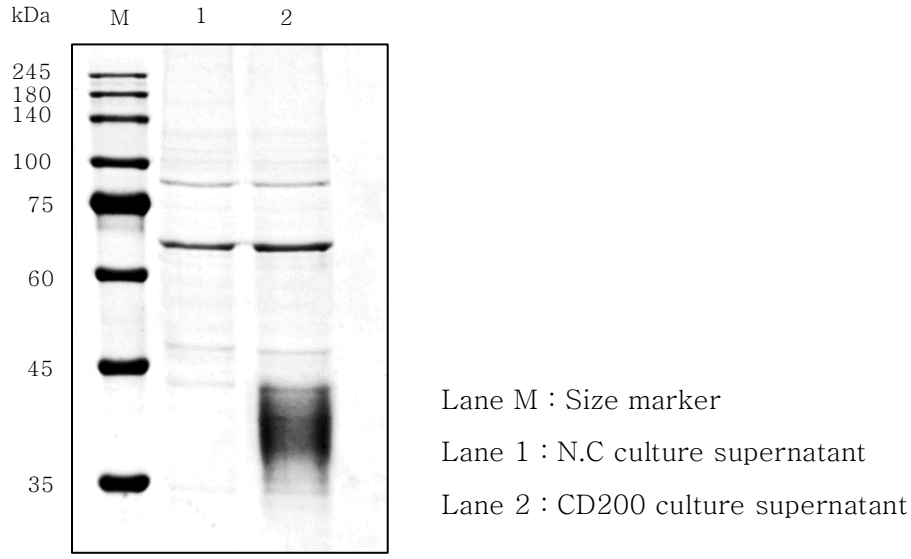



**Figure 9. Expression of CD200 confirmed by comparing the culture supernatant to a negative control using Coomassie Brilliant Blue staining.** 10 $\mu$l of sample was applied to each lane. Through comparison with negative control media, the background band (Lane 1) that appears during HEK293F cell culture was identified, emphasizing typical characteristics. Protein expression was evident, and the protein band appeared smeared, attributed to glycosylation and distribution of N-glycans during CD200 expression.

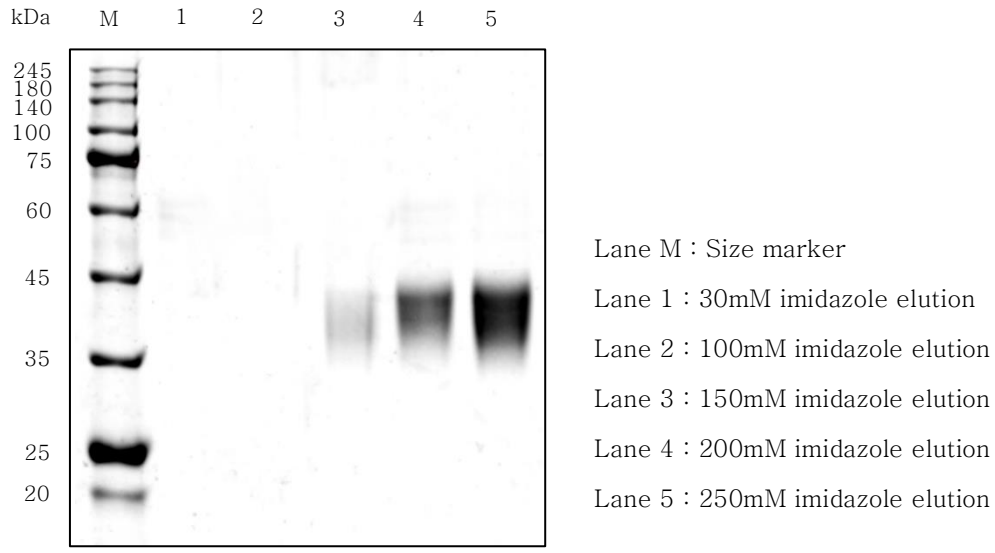
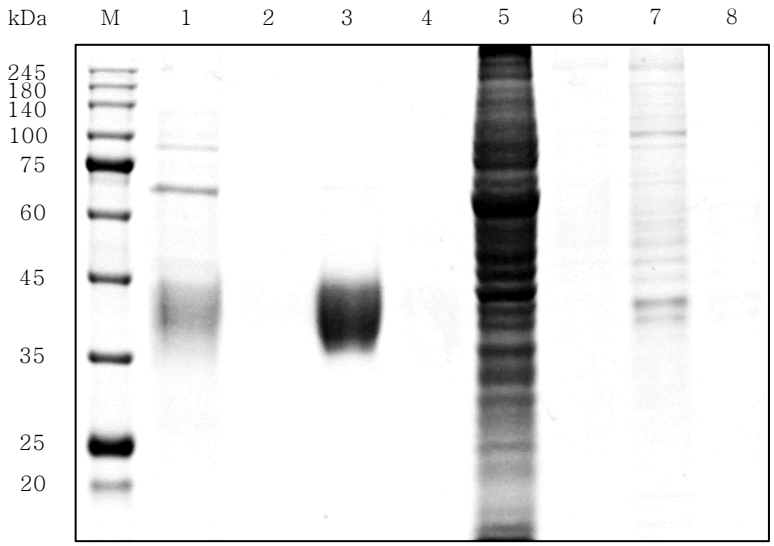



**Figure 10. Results of elution obtained by dividing the imidazole concentration by 30-250 mM using Coomassie Brilliant Blue staining.** 10 $\mu$l of sample was applied to each lane. Proteins were eluted at concentrations of imidazole of 30, 100, 150, 200, and 250 mM. Therefore, 250 mM imidazole, which shows the highest concentration and the darkest band, was selected as the elution buffer condition.




Lane M : Size marker

Lane 1 : CD200 culture supernatant

Lane 2 : media elution fraction 1

Lane 3 : media elution fraction 2

Lane 4 : media elution fraction 3

Lane 5 : CD200 culture lysate

Lane 6 : lysate elution fraction 1

Lane 7 : lysate elution fraction 2

Lane 8 : lysate elution fraction 3

**Figure 11. Purified CD200 confirmed by Coomassie Brilliant Blue staining.** 10 $\mu$l of sample was applied to each lane. The amount of chromatographic resin was 1 ml, and the elution was also eluted in fractions of 1 ml from the column. After flowing the elution buffer as much as the resin, protein was detected in the second fraction (Lane 3). As almost no protein was detected in the lysate elution (Lane 7), it was confirmed that protein secretion was performed well.

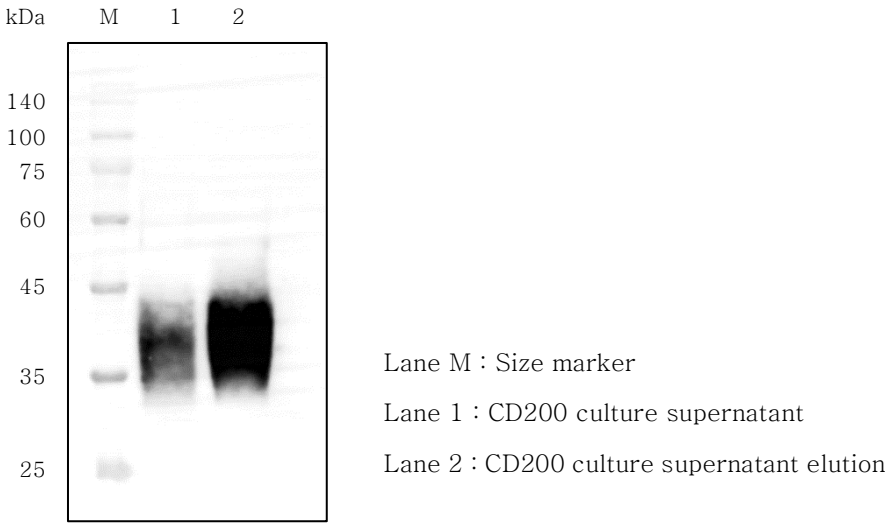
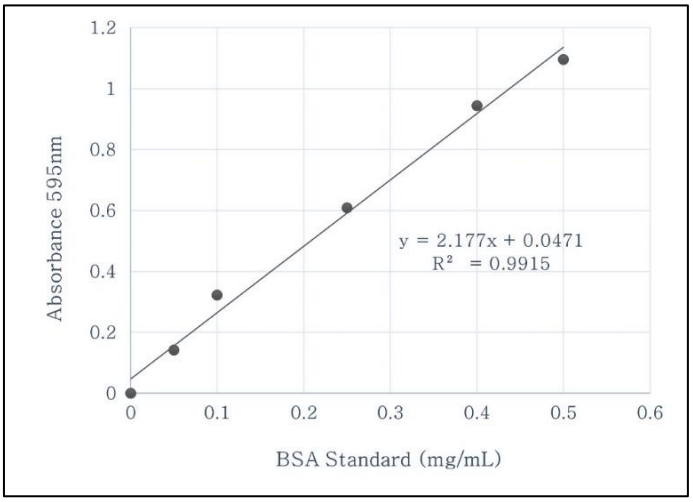



**Figure 12. Western blot for identification of expressed proteins.** After using an anti-histidine, HRP conjugated antibody (Sigma-Aldrich®), it was determined that both samples contained the necessary CD200 recombinant protein.

## 3. CD200 quantification and concentration

The purified CD200 was quantified using Bradford assay. The concentration of CD200 was calculated using a standard curve with BSA concentrations ranging from 0.05 mg/ml to 0.5 mg/ml as the standard. (Figure 13.) The calibration curve of the standard curve expressed linearly was $y=2.177x+0.0471$, where x represents the concentration of the sample and y represents the absorbance. The reliability of the calibration curve is expressed as an $R^2$ (coefficient of determination) between 0 and 1. In this case, the $R^2$ value was greater than 0.99, so it can be considered a reliable result. The OD value of CD200 was found to be 0.725, and the protein concentration was calculated by substituting this value into the above calibration curve equation. As a result, the concentration of CD200 was confirmed to be 0.311 mg/ml (1 ml), and the amount secured was 0.311 mg. Proteins were concentrated to secure the amount of protein required for the next step, biopanning. It was concentrated 10-fold through an Amicon® Centrifugal Filter, and 3 mg of protein was obtained per fraction.




| mg/ml | OD 595nm |
|---|---|
| 0 | 0 |
| 0.05 | 0.142 |
| 0.1 | 0.322 |
| 0.25 | 0.609 |
| 0.4 | 0.944 |
| 0.5 | 1.096 |
| purified CD200 | 0.725 |

**Figure 13. The Bradford assay standard curve for measuring CD200 concentration.** The calibration curve of the standard curve was calculated as y=2.177x+0.0471. The concentration was calculated by applying the absorbance of purified CD200 to the formula and was determined to be 0.311 mg/ml.

## 4. Measurement of protein impurities by Protein MS

To assess impurities and determine the size of the purified protein eluate, three experiments were conducted: Size exclusion chromatography (SEC), Reverse-phase chromatography (RP-HPLC), and Intact mass spectrometry (Intact MS).

### 1) Size exclusion Chromatography (SEC)

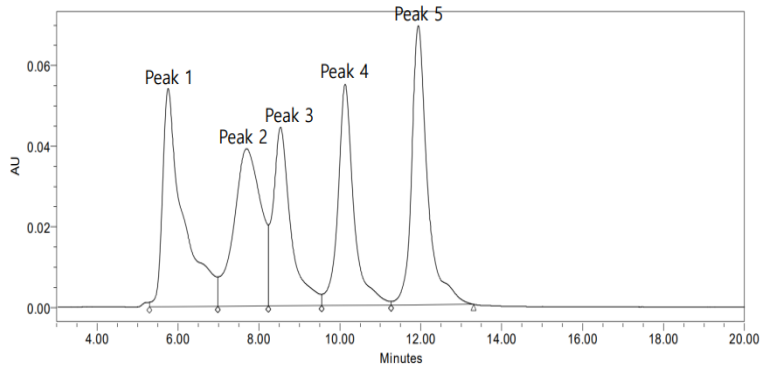
SEC was employed to confirm protein aggregation and separate protein samples based on size. Retention times were established for each size using five size markers. Thyroglobulin, bovine (670 kDa), $\gamma$-globulin, bovine (158 kDa), Ovalbumin, chicken (44 kDa), Myoglobin, horse (17 kDa), and Vitamin B12 (1.35 kDa) were detected in order of increasing size. (Figure 14.) Herceptin, a commercial antibody used as a control, displayed a sharp peak at a retention time appropriate for its size (~148 kDa). (Figure 15(a).) In contrast, CD200 exhibited a very low absorbance value, and the peak was broad, indicating imperfect separation during analysis. (Figure 15(b).) The incomplete separation observed during the analysis can be attributed to glycosylation, a phenomenon that occurs during the expression of CD200.

2) Reverse-phase chromatography (RP-HPLC)

Reverse-phase chromatography was employed to measure protein impurity. As with SEC, Herceptin was used as a control. (Figure 16(a).) Both Herceptin and CD200 displayed sharp, single peaks, confirming low impurity and high purity for both proteins. (Figure 16(b).) In RP-HPLC, proteins with low hydrophobicity are detected first, and the retention time of CD200 was shorter than that of Herceptin, suggesting that CD200 is a less hydrophobic protein.

3) Intact Mass Spectrometry

SDS-PAGE analysis revealed that the size of CD200(40-45 kDa) appeared larger than that of CD200 (extracellular region, 23.8 kDa) due to the glycosylation. (Figure 11.) Following treatment with PNGase F to remove N-glycans, the molecular weight was measured using Intact MS. (Figure 17.)

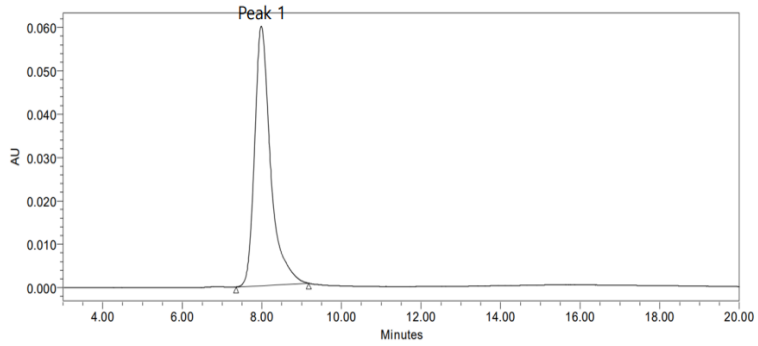



| Peak | Retention time | Peak area | % Area | Height |
|---|---|---|---|---|
| 1 | 5.752 | 1912459 | 22.52 | 54157 |
| 2 | 7.693 | 1825119 | 21.49 | 39068 |
| 3 | 8.534 | 1365633 | 16.08 | 44310 |
| 4 | 10.130 | 1498898 | 17.65 | 54867 |
| 5 | 11.940 | 1890806 | 22.26 | 69258 |

Figure 14. Size-markers for SEC analysis.

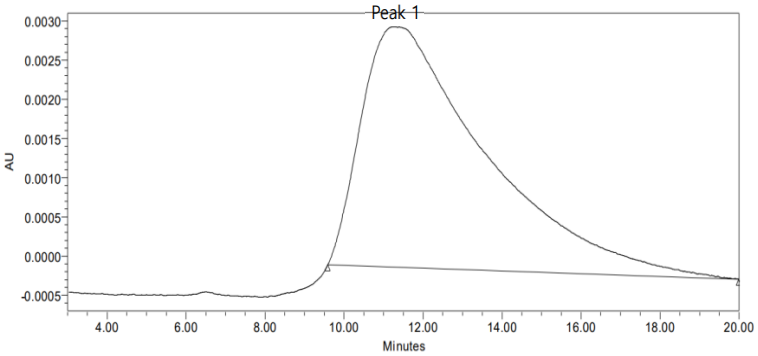
In order of peak 1-5, Thyroglobulin, bovine (670 kDa), gamma-globulin, bovine (158 kDa), Ovalbumin, chicken (44 kDa), Myoglobin, horse (17 kDa), Vitamin B12 (1.35 kDa).

a.




| Peak | Retention time | Peak area | % Area | Height |
|---|---|---|---|---|
| 1 | 7.986 | 1673826 | 100.0 | 59910 |

b.

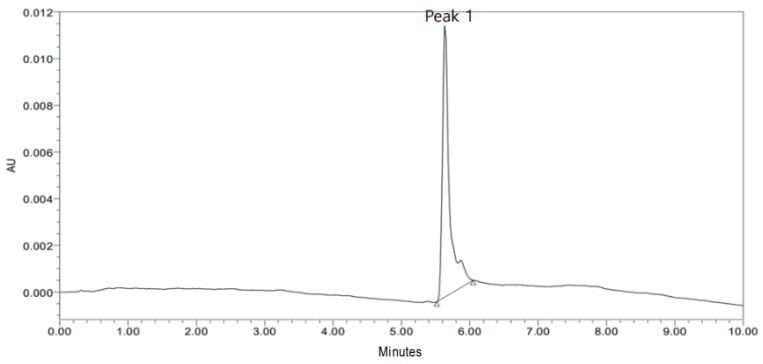



| Peak | Retention time | Peak area | % Area | Height |
|---|---|---|---|---|
| 1 | 11.232 | 671242 | 100.0 | 3066 |

**Figure 15. Size-exclusion chromatography analysis results.**

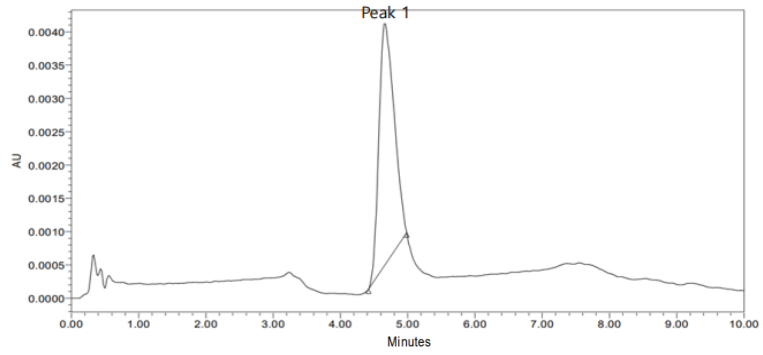
a. Herceptin 20 ㎍, commercial antibody used as control (~148 kDa). b. CD200 19.25 ㎍ (extracellular region, 23.8 kDa). The size-exclusion chromatography analysis of CD200 reveals an extremely low absorbance value and a broadly spread peak, indicating inadequate separation of fragments during the analysis. This suggests that the separation was compromised due to glycosylation occurring during the expression of CD200.

a.




| Peak | Retention time | Peak area | % Area | Height |
|---|---|---|---|---|
| 1 | 5.639 | 82839 | 100.0 | 11571 |

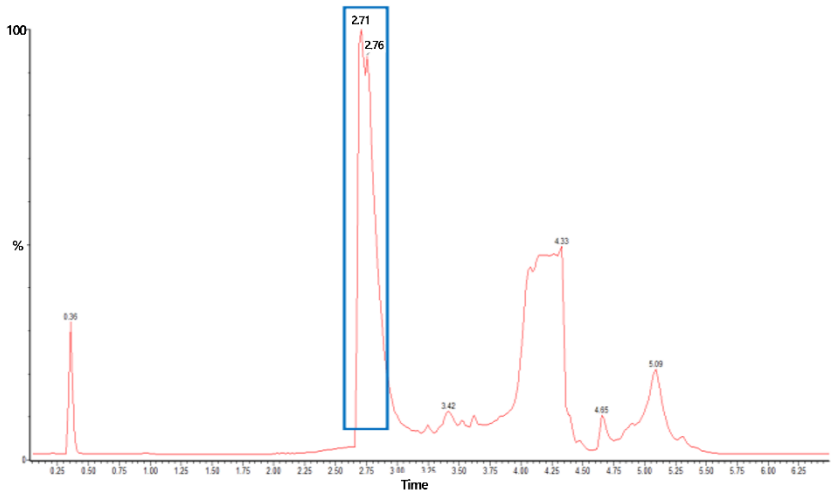
b.

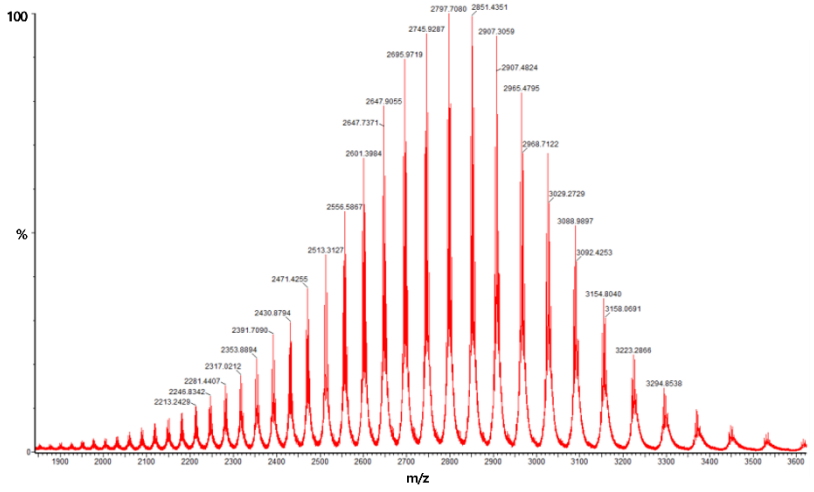


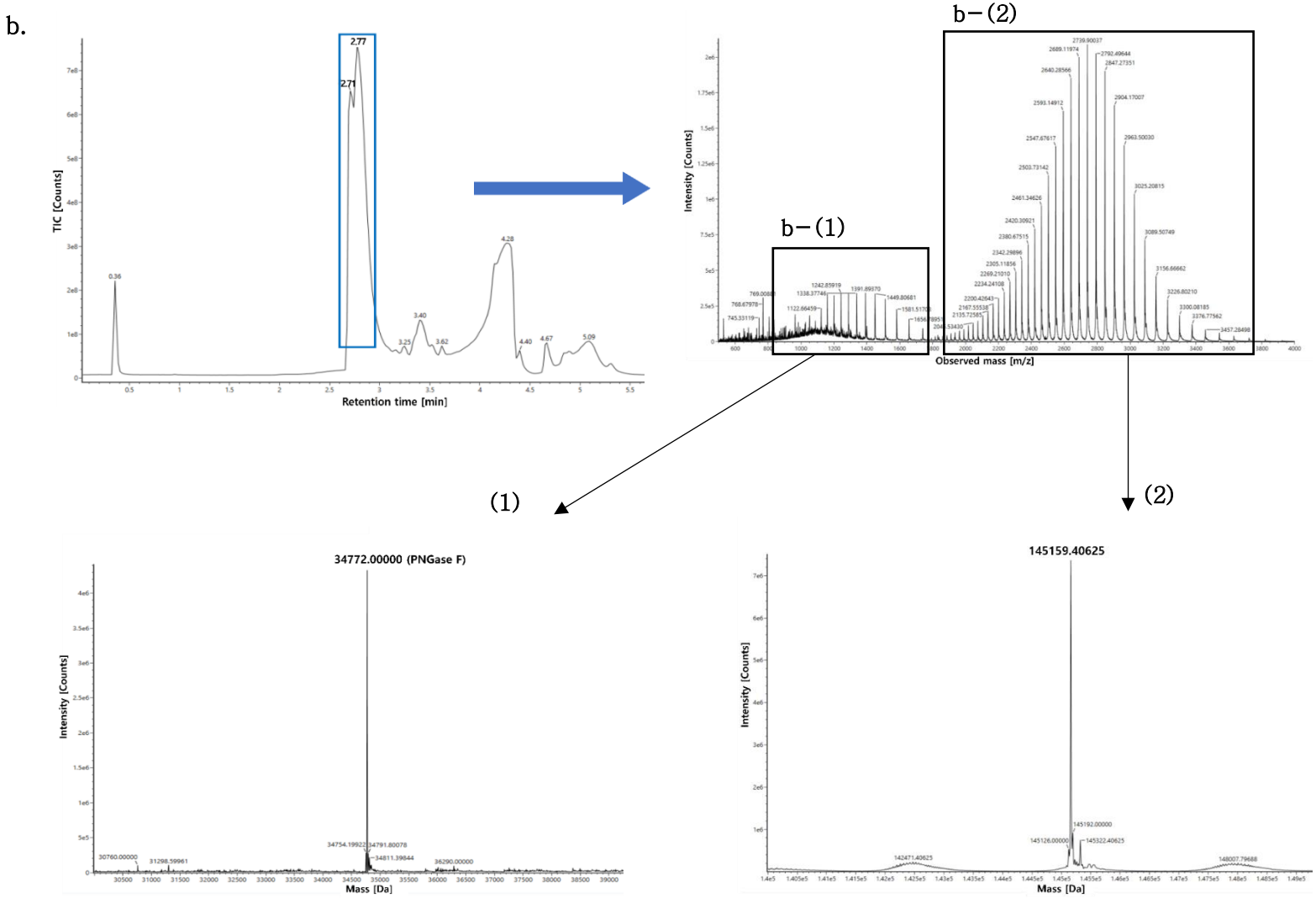

| Peak | Retention time | Peak area | % Area | Height |
|---|---|---|---|---|
| 1 | 4.660 | 56582 | 100.0 | 3639 |

**Figure 16. Reverse-phase chromatography analysis results.** a. Herceptin 3 μg, commercial antibody used as control. b. CD200 9.63 μg. The reversed-phase chromatographic analysis of CD200 revealed a sharp single peak that was clearly detected, indicating very low protein impurities.

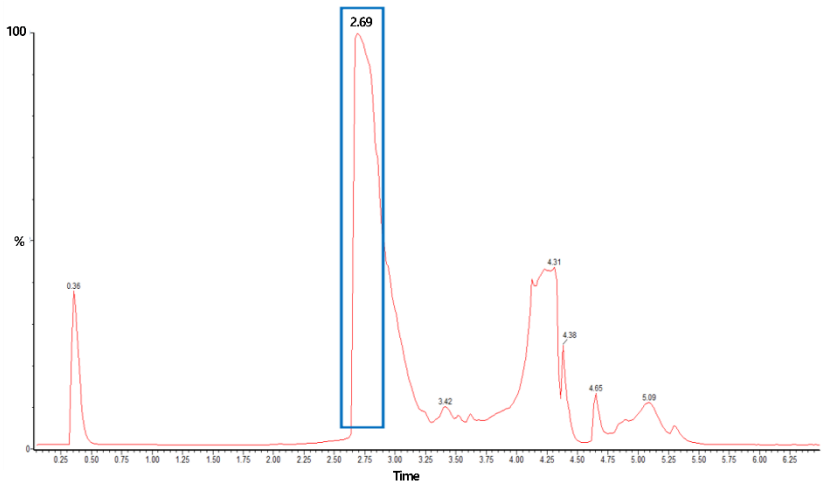
a.

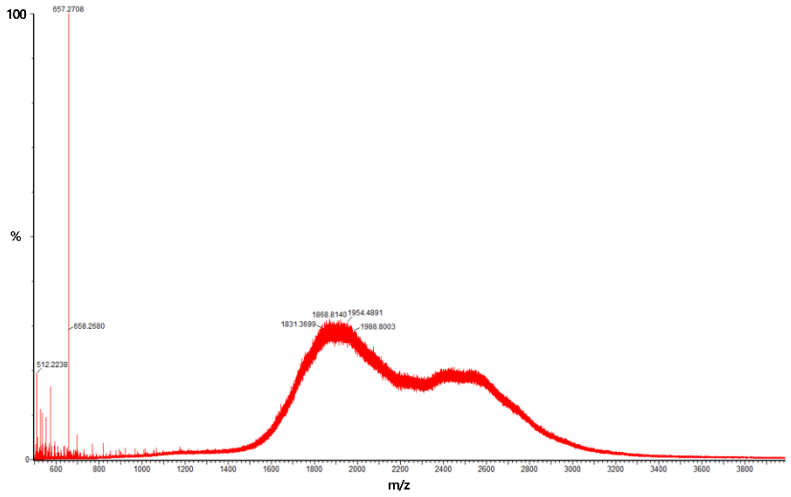

100
%
0
2.71
2.76
0.36
3.42
4.33
4.65
5.09
Time


100
%
0
2797.7080
2851.4351
2745.9287
2907.3059
2695.9719
2907.4824
2965.4795
2647.9055
2647.7371
2968.7122
2601.3984
3029.2729
2556.5867
3088.9897
2513.3127
3092.4253
2471.4255
3154.8040
2430.8794
3158.0691
2391.7090
3223.2866
2353.8894
3294.8538
2317.0212
2281.4407
2246.8342
2213.2429
1900
2000
2100
2200
2300
2400
2500
2600
2700
2800
2900
3000
3100
3200
3300
3400
3500
3600
m/z


b.
TIC [Counts]
Retention time [min]
2.77
2.71
0.36
3.40
3.25
3.62
4.28
4.40
4.67
5.09
b−(2)
b−(1)
Intensity [Counts]
Observed mass [m/z]
(1)
(2)
34772.00000 (PNGase F)
Intensity [Counts]
Mass [Da]
30760.00000
31298.59961
34754.19922
34791.80078
34811.39844
36290.00000
145159.40625
Intensity [Counts]
Mass [Da]
145192.00000
145126.00000
145322.40625
142471.40625
148007.79688

C.


100
%
0
2.69
0.36
3.42
4.31
4.38
4.65
5.09
Time


100
%
0
657.2708
658.2680
512.2238
1868.8140
1954.4891
1831.3699
1988.8003
m/z

d.

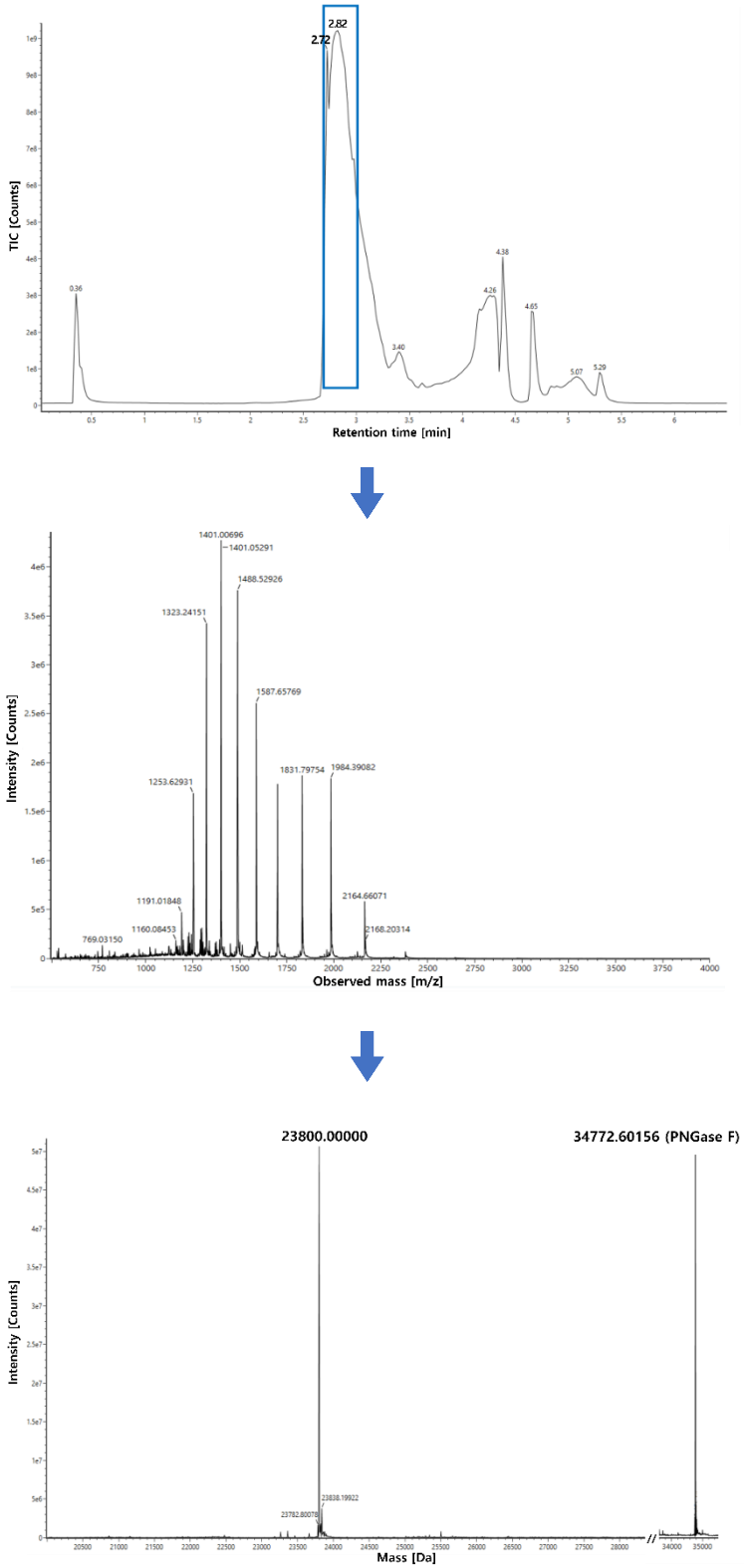

TIC [Counts]
Retention time [min]
2.82
2.72
0.36
3.40
4.26
4.38
4.65
5.07
5.29
Intensity [Counts]
Observed mass [m/z]
1401.00696
1401.05291
1488.52926
1323.24151
1587.65769
1831.79754
1984.39082
1253.62931
1191.01848
1160.08453
769.03150
2164.66071
2168.20314
23800.00000
34772.60156 (PNGase F)
Mass [Da]

**Figure 17. Intact mass analysis results.** a. Herceptin, commercial antibody used as control, 0.15 μg (w/o PNGase F). b. Herceptin 0.15 μg (w/ PNGase F), b-(1) PNGase F, b-(2) Herceptin. c. CD200 0.4 μg (w/o PNGase F). d. CD200 0.38 μg (w/ PNGase F).

In the Intact MS results of Herceptin treated with PNGase F, two sizes were observed: PNGase F (b-(1)) and Herceptin (b-(2)). The same observation was made for the Intact MS results of CD200 treated with PNGase F (d). Based on the size of the extracellular region of CD200, which is 23.8 kDa, these results suggest the successful removal of all glycosylation sites.

## 5. Phage extraction that selectively binds to CD200

Phage display method was employed to selectively extract phages with specific binding to CD200. To achieve this, a 12-mer phage display library was bound to CD200 immobilized on a 96-well plate. The process involved three rounds of affinity selection, where a phage library with a concentration of $2 \times 10^{11}$ pfu/ml underwent processing. Only strongly bound phages were eluted and subsequently amplified. After the phages were eluted, the resulting solution was used to infect E. coli cultured to mid-log phase (OD = 0.5), and the phage count was determined through blue/white selection on an IPTG/X-gal plate.

An issue arose during this process, wherein the number of bald phages—phages lacking displayed peptides—increased as the rounds progressed. To mitigate the progression of bald phages to the next round, amplification was selectively omitted in each round. Various conditions were tested to identify the most effective method: 1) omitting amplification only in the first round, 2) omitting amplification only in the second round and 3) omitting amplification in both the first and second rounds. After

experimentation, the most effective method－omitting amplification only in the second round－was adopted. Consequently, it was observed that the ratio of phages selectively binding to CD200 increased with each successive round. (Table 6.)

Table 6. Titer of phage selectively bound to CD200.

As a result of performing three rounds of biopanning to extract phages that selectively bind to CD200, it was confirmed that the Output/Input ratio of phage increased.

| Round | Input (pfu/ml) | Output (pfu/ml) | Recovery* (%) |
|---|---|---|---|
| #1 | $2 \times 10^{11}$ | $6 \times 10^{5}$ | 0.0003 |
| #2 | $2 \times 10^{11}$ | $1 \times 10^{7}$ | 0.005 |
| #3 | $1 \times 10^{6}$ | $1 \times 10^{4}$ | 1 |

* Recovery is given as Output/Input

## 6. Peptide sequence selection through Phage display

Following the third round of panning, blue plaques were selected and prepared for phage DNA extraction, resulting in the acquisition of phage single-stranded DNA (ssDNA). Subsequent analysis of the DNA sequences revealed classification into seven distinct peptide sequences. The obtained peptide sequences were listed based on their frequency of occurrence. (Table 7.) Among the 14 sequence analyses conducted, the CFAGTPSILMLA sequence was identified in five phage clones, constituting approximately 36%, followed by the GVLNSSPSTRFV sequence, accounting for about 21%.

Table 7. Peptide sequences of phages obtained after 3 rounds of panning.

The analyzed DNA sequences of the selected phage clones and the number of times each sequence appears are shown.

| Peptide sequence | Frequency |
|---|---|
| CFAGTPSILMLA | 5 |
| GVLNSSPSTRFV | 3 |
| VSVPGIITGTLR | 2 |
| YIPLGSPTPRSM | 1 |
| HGASYASMTVDN | 1 |
| VVGRAMAYSTIP | 1 |
| WSAVIPPVSKVL | 1 |

# IV. DISCUSSION

The interaction between CD200 and CD200R is implicated in various immune diseases. To intervene in this interaction, the study aimed to identify a peptide binding to CD200 and select a method to obstruct this binding. CD200, required for phage display, was expressed using a mammalian expression system (HEK cell) and obtained through nickel affinity chromatography.

Phage display is known to be an effective technology that can selectively extract variants that bind to target proteins and classify variants consisting of peptides with random sequences into various sets. Positive phage clones, binding to CD200, were identified through screening the phage display library. Sequence analysis of these phage clones from a random 12-mer phage display library revealed seven amino acid sequences.

Future endeavors will involve performing SPR to determine the binding affinity between CD200 and proteins selectively extracted through biopanning. Additionally, it is important to synthesize peptides based on the amino acid sequences of the obtained phage clones and evaluate their efficacy in binding CD200 and inhibiting CD200-

CD200R interaction. These additional studies hold the potential to synthesize selective and potent peptides against CD200, with further prospects for the development of peptide inhibitors for the CD200–CD200R interaction.

# V. CONCLUSION

This study aimed to identify peptide inhibitors binding to CD200 through the phage display technology. The necessary CD200 recombinant protein was successfully expressed and purified, ensuring an adequate quantity for phage display. Following three rounds of biopanning, phage plaques were obtained, purified, and ssDNA was extracted. Sequencing of 14 plaques revealed two sequences with the highest frequency: CFAGTPSILMLA and GVLNSSPSTRFV.

These findings suggest the potential discovery of a peptide capable of binding to CD200 and inhibiting the CD200-CD200R interaction. Furthermore, there is optimism regarding the development of a peptide inhibitor that can robustly and selectively hinder this interaction.

# REFERENCE

[1] M. Howard (Jack) West, "Immune Checkpoint Inhibitors," *JAMA Oncol*, vol. 1, no. 1, p. 115, Mar. 2015.

[2] G. J. Wright *et al.*, "Lymphoid/Neuronal Cell Surface OX2 Glycoprotein Recognizes a Novel Receptor on Macrophages Implicated in the Control of Their Function," *Immunity*, vol. 13, pp. 233–242, 2000.

[3] T. P. Rygiel and L. Meyaard, "CD200R signaling in tumor tolerance and inflammation: A tricky balance," *Current Opinion in Immunology*, vol. 24, no. 2. pp. 233–238, Apr. 2012.

[4] F. M. Wurm, "Production of recombinant protein therapeutics in cultivated mammalian cells," *Nat Biotechnol*, vol. 22, no. 11, pp. 1393–1398, Nov. 2004.

[5] K. H. Khan, "Gene expression in mammalian cells and its applications," *Adv Pharm Bull*, vol. 3, no. 2, pp. 257–263, 2013.

[6] M. Hunter, P. Yuan, D. Vavilala, and M. Fox, "Optimization of Protein Expression in Mammalian Cells," *Curr Protoc Protein Sci*, vol. 95, no. 1, Feb. 2019.

[7] Alain R. Bernard, *Production of Proteins by Transient*

*Expression*. cell culture technology for pharmaceutical and cell-based therapies, 2005.

[8] S. C. Almo and J. D. Love, "Better and faster: improvements and optimization for mammalian recombinant protein production," *Curr Opin Struct Biol*, vol. 26, pp. 39-43, Jun. 2014.

[9] P. L. Pham *et al.*, "Large-scale transient transfection of serum-free suspension-growing HEK293 EBNA1 cells: Peptone additives improve cell growth and transfection efficiency," *Biotechnol Bioeng*, vol. 84, no. 3, pp. 332-342, Nov. 2003.

[10] L. Ledsgaard, M. Kilstrup, A. Karatt-Vellatt, J. McCafferty, and A. H. Laustsen, "Basics of antibody phage display technology," *Toxins*, vol. 10, no. 6. MDPI AG, Jun. 09, 2018.

[11] E. M. Zygiel *et al.*, "Various mutations compensate for a deleterious lacZ$\alpha$ insert in the replication enhancer of M13 bacteriophage," *PLoS One*, vol. 12, no. 4, Apr. 2017.

[12] J. Wang *et al.*, "In vitro knockdown of TsDNase II-7 suppresses Trichinella spiralis invasion into the host's intestinal epithelial cells," *PLoS Negl Trop Dis*, vol. 17, no. 6, Jun. 2023.

[13] M. Fussenegger, "Genetic optimization of recombinant glycoprotein production by mammalian cells," *Trends Biotechnol*,

vol. 17, no. 1, pp. 35-42, Jan. 1999.

[14] D. Hatherley, S. M. Lea, S. Johnson, and A. N. Barclay, "Structures of CD200/CD200 receptor family and implications for topology, regulation, and evolution," *Structure*, vol. 21, no. 5, pp. 820-832, May 2013.

[15] S. Kühn and P. F. Zipfel, "The baculovirus expression vector pBSV-8His directs secretion of histidine-tagged proteins," *Gene*, vol. 162, no. 2, pp. 225-229, Sep. 1995.

[16] W. Xia *et al.*, "High levels of protein expression using different mammalian CMV promoters in several cell lines," *Protein Expr Purif*, vol. 45, no. 1, pp. 115-124, Jan. 2006.

[17] R. Vazquez-Lombardi, D. Nevoltris, A. Luthra, P. Schofield, C. Zimmermann, and D. Christ, "Transient expression of human antibodies in mammalian cells," *Nat Protoc*, vol. 13, no. 1, pp. 99-117, Jan. 2018.

[18] J. E. Nettleship *et al.*, "A pipeline for the production of antibody fragments for structural studies using transient expression in HEK 293T cells," *Protein Expr Purif*, vol. 62, no. 1, pp. 83-89, Nov. 2008.

[19] A. Yeliseev *et al.*, "Thermostability of a recombinant G

protein-coupled receptor expressed at high level in mammalian cell culture," *Sci Rep*, vol. 10, no. 1, p. 16805, Oct. 2020.

[20] M. Lipsitch, E. Tchetgen Tchetgen, and T. Cohen, "Negative Controls: A Tool for Detecting Confounding and Bias in Observational Studies," *Epidemiology*, vol. 21, no. 3, pp. 383-388, May 2010.

[21] E. Englesberg and G. Wilcox, "REGULATION: POSITIVE CONTROL," *Annu Rev Genet*, vol. 8, no. 1, pp. 219-242, 1974.

[22] S. Hober, K. Nord, and M. Linhult, "Protein A chromatography for antibody purification," *Journal of Chromatography B*, vol. 848, no. 1, pp. 40-47, Mar. 2007.

[23] M. Zarrineh, I. S. Mashhadi, M. Farhadpour, and A. Ghassempour, "Mechanism of antibodies purification by protein A," *Anal Biochem*, vol. 609, p. 113909, Nov. 2020.

[24] Kevin J. Petty, *Metal-Chelate Affinity Chromatography*, vol. 36. Current Protocols in Molecular Biology, 1996.

[25] J. S. Kim, C. A. Valencia, R. Liu, and W. Lin, "Highly-Efficient Purification of Native Polyhistidine-Tagged Proteins by Multivalent NTA-Modified Magnetic Nanoparticles," *Bioconjug*

*Chem*, vol. 18, no. 2, pp. 333–341, Mar. 2007.

[26] S. Knecht, D. Ricklin, A. N. Eberle, and B. Ernst, "Oligohis-tags: mechanisms of binding to $Ni^{2+}$–NTA surfaces," *Journal of Molecular Recognition*, vol. 22, no. 4, pp. 270–279, Jul. 2009.

[27] F. B. Bjϕrkskov *et al.*, "Purification and functional comparison of nine human Aquaporins produced in Saccharomyces cerevisiae for the purpose of biophysical characterization," *Sci Rep*, vol. 7, no. 1, p. 16899, Dec. 2017.

[28] J. M. Manns, "SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Proteins," *Curr Protoc Microbiol*, vol. 22, no. 1, Aug. 2011.

[29] W. Gao and E. Goldman, "Use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis to resolve mRNA and its protein product in one gel," *The FASEB Journal*, vol. 11, no. 13, pp. 1153–1156, Nov. 1997.

[30] S. Roy and V. Kumar, "A Practical Approach on SDS PAGE for Separation of Protein," *International Journal of Science and Research*, 2014.

[31] X. Zhao *et al.*, "Transient expression of Fc–fused

human glycoprotein 130 in Expi293F suspension cells," *Protein Expr Purif*, vol. 124, pp. 41–47, Aug. 2016.

[32] M. M. Brysk, E. Barlow, T. Bell, S. Rajaraman, and R. W. Stach, "Purification of Proteins from Polyacrylamide Gels, Free of Detergent or Dye," *Prep Biochem*, vol. 18, no. 2, pp. 217–225, Jun. 1988.

[33] J. Gibbons, "Western blot: Protein transfer overview," *N Am J Med Sci*, vol. 6, no. 3, p. 158, 2014.

[34] Lita A. Freeman, "Western blots," in *Lipoproteins and Cardiovascular Disease: Methods and Protocols*, vol. 1027, pp. 369–385, 2013.

[35] A. Alegria-Schaffer, A. Lodge, and K. Vattem, "Chapter 33 Performing and Optimizing Western Blots with an Emphasis on Chemiluminescent Detection," pp. 573–599, 2009.

[36] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Anal Biochem*, vol. 72, no. 1–2, pp. 248–254, May 1976.

[37] O. Ernst and T. Zor, "Linearization of the Bradford Protein Assay," *Journal of Visualized Experiments*, no. 38, Apr.

2010.

[38] M. Loh, P. Shian, F. Adibah, and A. Majid, "The Performance of Different Pre-Treatment Techniques in the Isolation and Purification of Monoclonal IgG Antibody," 2006.

[39] P. Hong, S. Koza, and E. S. P. Bouvier, "A REVIEW SIZE-EXCLUSION CHROMATOGRAPHY FOR THE ANALYSIS OF PROTEIN BIOTHERAPEUTICS AND THEIR AGGREGATES," *J Liq Chromatogr Relat Technol*, vol. 35, no. 20, pp. 2923-2950, Jan. 2012.

[40] E. Lemasson, S. Bertin, P. Hennig, E. Lesellier, and C. West, "Impurity profiling of drug candidates: Analytical strategies using reversed-phase and mixed-mode high-performance liquid chromatography methods," *J Chromatogr A*, vol. 1535, pp. 101-113, Feb. 2018.

[41] P. Tit-oon *et al.*, "Intact mass analysis reveals the novel O-linked glycosylation on the stalk region of PD-1 protein," *Sci Rep*, vol. 13, no. 1, p. 9631, Jun. 2023.

# 국문요약

면역관문 억제제 (Immune checkpoint inhibitor)는 T 세포나 종양세포에 발현되는 면역관문을 구성하는 단백질의 기능을 차단하는 약물로, 이는 체크포인트 단백질 억제제의 일종이다. 체크포인트 단백질 억제제는 T 세포가 암세포를 죽이도록 도와 잠재적인 항암 요법에 사용될 수 있다. CD200 (OX-2 membrane glycoprotein)은 면역 체크포인트의 흥미로운 표적이 될 수 있다. CD200 단백질과 그의 리셉터인 CD200R의 상호작용은 알레르기 질환, 감염, 관절염, 이식과 같은 면역 관련 질환, 다발성 경화증 및 전신성 홍반성 루푸스와 같은 자가면역 질환과 관련되어 있다. CD200과 CD200R의 결합을 차단하기 위하여, CD200에 결합하는 항-CD200 펩타이드를 스크리닝 하는 실험을 설계했다. 우선 CD200 단백질을 얻기 위해 포유류 단백질 발현 시스템을 이용하여 단백질을 발현하였고, 고효율 발현을 위해 HEK (Human embryonic kidney) 세포를 사용하였다. 발현된 단백질의 정제를 위해 히스티딘 태그를 이용한 니켈 친화성 크로마토그래피를 수행하였고, Protein MS를 진행하여 단백질의 불순도를 확인하였다. 이후, CD200 단백질에 특이적으로 결합하는 펩타이드를 스크리닝하기 위해 -주어진 표적 분자에 대한 결합 친화도를 기반으로 가장 흥미로운 후보를 인식할 수 있는 스크리닝 기술인- 파지 디스플

레이를 수행했다. 3번의 바이오패닝을 거쳐서 얻은 파지 플라크를 증폭하고 정제한 후, DNA 시퀀싱을 진행하여 12-mer 펩타이드 서열을 확인하였다. 추후 분석을 통해 선별되는 서열이 CD200과 CD200R의 상호작용을 차단하는 길항제로서의 가능성을 가지고 있을 것으로 사료된다.