Peptide-based immunoabsorbents: molecular grafting of IgG-Fc -binding epitopes of Protein A onto a *de novo*-designed helix-loop-helix peptideKumiko Kawabata^a, Hirokazu, Nagai^b, Nao Konishi^a, Daisuke Fujiwara^a, Ryo Sasaki^b, Takafumi Ichikawa^b, and Ikuo Fujii^{a,*}^aDepartment of Biological Science, Graduate School of Science, Osaka Prefecture University, 1-1, Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan^bASAHIKASEI MEDICAL CO., LTD, 1-105 Kanda Jinbocho, Chiyoda-ku, Tokyo 101-8101, Japan

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ABSTRACT

The development of immunoabsorbents that have high specificity for immunoglobulin and no immunogenicity is essential for immunoabsorption treatment of autoimmune diseases. In this study, we designed peptide immunoabsorbents by molecular grafting of the IgG-Fc binding epitopes of Protein A onto a *de novo*-designed helix-loop-helix peptide. Linear (linG7A5) and cyclic (cyG7A5) grafted peptides were synthesized to test their binding affinity and specificity. Peptide cyG7A5 demonstrated high specificity for human IgG-Fc, with a K_D of 19 μ M, and demonstrated no affinity to other plasma proteins, human serum albumin, or fibrinogen. To evaluate their immunoabsorbance efficiency, the grafted peptides and Protein A were conjugated to polyvinyl acetate resin and tested in a batch-wise process for adsorption removal of IgG from human plasma. The IgG capture capacities of the peptides correlated well with their binding affinities. Interestingly, cyG7A5 showed a higher binding specificity for IgG than did Protein A.

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1. Introduction

Overexpression of immunoglobulin (Ig) results in a severe inflammatory response in patients with proven or suspected autoimmune disease, such as myasthenia gravis, autoimmune neuropathy, Guillain-Barre syndrome, or systemic lupus erythematosus. The removal of Ig has been shown to be an effective therapeutic method for treating such diseases. Immunoabsorption is recognized as a more useful and safe therapeutic method than plasma exchange, which involves removal of all plasma proteins, such as albumin and blood clotting factors.^{1,2}

Currently, two different types of Ig adsorbents are available for clinical use: biological and non-biological adsorbents. Protein A and anti-Ig antibodies are representative examples of biological adsorbents.³⁻⁷ Although such biological adsorbents have high specificity for IgG, they have to be purified from biological sources, which poses potential infection risks due to cross-contamination.⁸ On the other hand, non-biological immunoabsorbents, such as tryptophan- and phenylalanine-conjugated resins are safer. However, in general, non-biological adsorbents exhibit lower affinity and are less selective than biological adsorbents.⁹ Therefore, we sought to develop a synthetic peptide adsorbent that combines the safety advantages of non-biological immunoabsorbents with the highly specific IgG-binding performance of biological immunoabsorbents.

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In a previous study, we designed a *de novo* helix-loop-helix (HLH) peptide, YT1-S, as a scaffold for conformationally constrained peptide ligands.^{10,11} The HLH peptide showed potential proteolytic resistance and non-immunogenicity, which are prerequisite properties for an immunoabsorbent. Amino acid residues located within the α -helices of Protein A are involved in binding to IgG-Fc. Therefore, in this work, we grafted the IgG-Fc-binding epitopes of Protein A onto two α -helices of the HLH peptide to generate a novel class of peptide-based immunoabsorbents. We then examined the binding affinity, specificity, and proteolytic resistance of the grafted HLH peptides. Furthermore, to evaluate the efficiency of the novel immunoabsorbents, we conjugated the grafted HLH peptides to a polyvinyl acetate (PVAc) resin to test batch-wise adsorption of IgG from human plasma.

2. Result and discussions

2.1. Design of the IgG-Fc-binding peptides

Protein grafting is a potential approach for imparting binding functionalities to proteins.¹²⁻¹⁵ Our HLH peptide is an excellent scaffold for protein grafting of epitopes using its α -helices. Protein A possesses an N-terminal flexible region (Ala¹-Lys⁷) and three α -helices (helix 1: Gln⁸-His¹⁸, helix 2: Asn²³-Asp³⁶, helix 3: Ser⁴¹-Ala⁵⁴).¹⁶ Protein A helices 1 and 2 reportedly interact with IgG-Fc, and helix 3 is essential to the global folding of Protein A in solution.^{17,18} Therefore, we grafted the binding epitopes of Protein A onto the YT1-S HLH peptide scaffold.

Figure 1. Design of the IgG-Fc-binding peptide cyG7A5 by protein grafting. (a) Schematic illustrating the design of grafted peptide cyG7A5 from Protein A. (b) Alignment of Protein A, cyG7A5, and YT-1S sequences. The three helices of Protein A are shown as underlined residues. Amino acids in red are grafted residues from protein A.

As shown in Figure 1, we designed to transfer the helix 1 epitopes (Phe¹³, Tyr¹⁴, Glu¹⁵, Ile¹⁶, Leu¹⁷ and His¹⁸) and helix 2 epitopes (Asn²³, Glu²⁴, Glu²⁵, Gln²⁶, Arg²⁷, Asn²⁸, Ala²⁹, Ile³¹, Ser³³, and Asp³⁶) of Protein A to the N-terminal and C-terminal helices of the YT1-S HLH peptide, respectively. Furthermore, based on the crystal structure of a complex of Protein A with IgG-Fc, an additional epitope was assumed to be Phe⁵, which was attached to the N-terminal end of the HLH scaffold with a spacer (Asn⁶, Met⁷, and Gln⁸). On the other hand, four alanine residues on the side face of the HLH peptide were replaced with hydrophilic residues (Arg¹¹, Arg¹², Lys³², and Arg³⁵) to increase the solubility in aqueous solution, because it was assumed that amino acids of these positions played no role in the binding from the crystal structure. Finally, the protein grafting provided a cyclic peptide, cyG7A5 (cyG7A5: NH₂-FNMQCQRRFYEILHEAAAAA-GGGGGGG-NEEORNAKIKSIRDDC-CONH₂).

To immobilize the grafted peptide on a resin for IgG adsorption or on a sensor chip for surface Plasmon resonance (SPR) measurement, we prepared a thiol peptide, cyG7A5C, in which a cysteine residue was introduced into the N-terminus of cyG7A5 (cyG7A5C: NH₂-CGFNMQCRRFYEILHEAAAAA-GGGGGGG-NEEQRNAIKSIRDDC-CONH₂). The corresponding linear peptide, linG7A5C, was also prepared as a reference to the cyclic peptide in examinations of the binding affinity, the proteolytic resistance, and the capacity of IgG adsorption. A cystein (Cys⁹) forming the intramolecular disulfide bond in cyG7A5 was replaced with a glutamine to provide a liner peptide, (linG7A5C: NH₂-FMQQQRRFYEILHEAAAAA-GGGGGGG-NEEQRNAIKSIRDDC-CONH₂).

2.2. Synthesis of cyG7A5, cyG7A5C and linG7A5C

The cyG7A5, cyG7A5C and linG7A5C peptides were synthesized by standard Fmoc-solid phase peptide synthesis using amide resin. Amino acid coupling was carried out using 1-[Bis(dimethylamino)methylene]-5-chloro-1*H*-benzotriazolium 3-oxidehexafluorophosphate (HCTU) coupling reagent.¹⁹ In the case of cyG7A5C, 1,3-thiazolidine-4-carboxylic acid (Thz)

was used for coupling to protect the thiol group of the N-terminal cysteine. After cleavage, the peptide was purified by RP-HPLC and characterized using MALDI-TOF-MS. The two cysteine residues of cyG7A5 and cyG7A5C were allowed to cross-link overnight in Tris-HCl (pH 8.5), respectively. After purification, the Thz group of cyG7A5C was deprotected by treatment with 0.2 M methoxyamine-HCl (pH < 4) to yield the free N-terminal cysteine.

2.3. CD spectra

To determine whether or not the grafted peptides adopt an α -helical conformation, we measured CD spectra for cyG7A5, linG7A5C, and cyG7A5C (Figure 2). Peptide linG7A5C exhibited double minima at 208 and 222 nm, indicating that the peptide has an α -helical structure in aqueous solution. Cyclic

Figure 2. CD spectra of cyG7A5, cyG7A5C, and linG7A5C. The peptides were dissolved in 20 mM phosphate buffer (pH 7.4) at 20 μ M.

Figure 3. Binding specificity analysis of cyG7A5C for human IgG-Fc. Peptide cyG7A5C was immobilized on CM5 sensor chip and human IgG-Fc, human whole IgG, Fibrinogen, HSA, BSA were injected at 1 μ M, respectively.

peptide cyG7A5C showed larger negative values of dual minima at 208 and 222 nm, suggesting that the cyclic peptide possesses more tight α -helical conformation than that of the linear peptide. These observations indicate that cyclization with the disulfide bond linkage between Cys⁹ and Cys³⁸ increases the conformational stability of the HLH peptides. In addition, both cyclic peptides cyG7A5 and cyG7A5C showed the almost same values of dual minima at 208 and 222 nm, suggesting, no influence of the additional cysteine residue to stability of α -helical structure.

2.4. Binding affinity and specificity for human IgG-Fc

The affinity of the grafted peptides for binding human IgG-Fc was examined using SPR analysis. Human IgG-Fc was immobilized onto a CM5 sensor chip by amine-coupling. Various concentrations of the peptides were injected to obtain sensorgrams, which were analyzed using the global fitting program, BIAevaluation 4.1. The dissociation constants (K_D) of cyG7A5, cyG7A5C, and linG7A5C for human IgG-Fc were 19,

Figure 4. Protease stability of cyG7A5C and linG7A5C. The reaction mixture for trypsin digestion contained 42 μ M of peptides, 240 μ g/L of trypsin, and 100 mM Tris-HCl buffer, pH = 8.5.

19, and 150 μ M, respectively. The affinity of linG7A5C for human IgG-Fc was 10-fold lower than that of the cyclic peptides, indicating that there is a parallel correlation between conformational stability and binding affinity.

Next, we defined the binding specificity for human IgG-Fc using SPR analysis. Peptide cyG7A5C was immobilized onto a CM5 sensor chip by thiol-coupling and its binding affinity for human IgG-Fc, IgG, fibrinogen, HSA, and BSA was examined. As shown in Figure 3, cyG7A5C showed high specificity for Ig, with affinity for human IgG-Fc and IgG but no affinity for the other plasma proteins.

2.5. Proteolytic resistance

A major problem with peptide therapeutic agents is their proteolytic sensitivity. In general, synthetic peptides have unfolded structures, which results in rapid enzyme degradation *in vivo*. However, because our grafted peptides assume an α -helical structure, they should resist proteolytic degradation. Therefore, we measured the susceptibility of the grafted peptides to trypsin-mediated degradation, as monitored by HPLC. Because the concentration of trypsin in healthy human serum is defined, ranging from 138 to 406 μ g/L²⁰, the peptides were incubated in a 240 μ g/L trypsin solution at 37°C. Both peptides were found to resist proteolytic degradation, with half-lives of 7.0 and 3.7 h for cyG7A5C and linG7A5C, respectively (Figure 4). As expected based upon its conformational stability, cyG7A5C was twice as resistant to proteolysis as linG7A5C. Considering that immunoadsorption therapy takes about 2 hours

Figure 5. Batchwise adsorption using peptide conjugated adsorbents. (a) Adsorption of IgG and fibrinogen (Fbg) from plasma on linG7A5C or cyG7A5C conjugated adsorbents. (b) Comparison between the capture capacity of IgG and the binding affinity of ligands.

(flow rate: 25–30 mL/min, volume: 2–3 L), the proteolytic resistance of our peptides indicates that they could be used as immunoabsorbents.

2.6. Batch-wise adsorption of human plasma IgG

To test the IgG-removal performance of the grafted peptides, linG7A5C and cyG7A5C were conjugated to a PVAc resin. Maleimido-activated PVAc resin was swelled in PBS and reacted separately with linG7A5C (51 mg, 11 mmol) and cyG7A5C (58 mg, 12 mmol) for 17 h to yield the corresponding peptide-immobilized gels (cyG7A5C: 3.8 μ mol/mL of gel, linG7A5C: 1.8 μ mol/mL of gel).

We used a batch-wise adsorption method to evaluate the IgG-adsorption performance of the peptide-conjugated adsorbents. Human plasma obtained from healthy blood donors was mixed with the conjugated PVAc gels (volume ratio of gel to plasma = 1:6) for 17.5 min at 37°C. The linG7A5C and cyG7A5C peptides removed 18 and 22% of the IgG, respectively (Figure 5 (a)). We performed the same test using Protein A-immobilized gel as a reference. Protein A, which has a high binding affinity for IgG-Fc (K_D = 20 nM), removed 39% of the IgG. As shown in Figure 5 (b), the IgG capture capacities of the peptides correlated well with their binding affinities.

The two grafted peptides and Protein A were also examined for batch-wise adsorption of another plasma component, fibrinogen. Peptide linG7A5C removed 11% of the fibrinogen. In contrast, peptide cyG7A5C, which showed a high specificity and high adsorption of IgG (22%), adsorbed fibrinogen only minimally (1.6%). Protein A removed 9% of the fibrinogen. Interestingly, the adsorption specificity (adsorption ratio; IgG:fibrinogen = 14:1) of cyG7A5C was much higher than that of Protein A (adsorption ratio; IgG:fibrinogen = 4:1).

3. Conclusion

In this study, we generated IgG-binding peptide, cyG7A5 by grafting the IgG-Fc binding epitopes of Protein A onto the HLH peptide scaffold. Immunoglobulin adsorbents were generated by conjugating the grafted peptides to PVAc resin. Using CD spectroscopy, we confirmed that peptide cyG7A5 has a stable α -helical conformation. SPR analysis indicated that cyG7A5 has specific affinity for IgG-Fc, and enzymatic degradation tests using trypsin indicated that the peptide is resistant to proteolysis. Therefore, cyG7A5 could potentially be used as an immunoabsorbent for extracorporeal therapy.

The results of SPR measurements and batch-wise adsorption experiments using plasma indicated that cyG7A5 has a high binding specificity for IgG-Fc. It is strongly expected that cyG7A5C-immobilized adsorbent will demonstrate better performance than non-biological adsorbents, which bind many plasma components other than IgG. Interestingly, the adsorption specificity of cyG7A5C was much higher than that of the biological adsorbent, Protein A. In batch-wise tests of the adsorption of IgG from human plasma, it was found that the capture capacity of the grafted peptides correlated well with their binding affinities. This suggests that improving the binding affinity of cyG7A5C further will provide a high-performance non-biological immunoabsorbent. This study provided promising examples for the development of highly selective adsorbents that could be used to target human IgG in the treatment of autoimmune diseases.

4. Materials and methods

4.1. Materials

HPLC-grade acetonitrile, acetic anhydride, diethylether, 1,2-ethanedithiol (EDT), and anisole were purchased from Nacalai

Tesque, Inc. (Kyoto, Japan). N,N-Dimethylformamide (DMF), piperidine, N,N-diisopropylethylamine (DIEA) for peptide synthesis, trifluoroacetic acid (TFA), and ethylmethylsulfide (EMS) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 1-[Bis (dimethylamino)methylene]-5-chloro-1H-benzotriazolium3-oxide hexafluorophosphate (HCTU) was purchased from Peptide Institute, Inc. (Osaka Japan). Written informed consent was obtained from all subjects, and the study was approved by the ethics committee at Asahikasei Medical Co., Ltd.

4.2. Peptide synthesis

All peptides were synthesized using Fmoc-PAL-PEG-PSTM amide resin (0.18 mmol/g loading). The cyG7A5 and linG7A5C peptides were synthesized in a stepwise manner at a 0.02- and 0.16-mmol scale, respectively, on an automated peptide synthesizer PSSM-8 (Shimazu). The cyG7A5C peptide was synthesized manually at a 0.5-mmol scale. All peptides were synthesized using an Fmoc solid-phase peptide synthesis strategy. A typical single coupling procedure was as follows: deprotection of the Fmoc group before each coupling was accomplished by treating with 20% piperidine/DMF (2 $\times$ 4 min). The subsequent Fmoc-protected amino acid was coupled for 30 min using Fmoc-amino acid (5 equiv.), HCTU (5 equiv.), and DIEA (10 equiv.). Acetylation of the α -amino group was accomplished by treating with 5% acetic anhydride/DMF. After synthesis was complete, the peptides were cleaved from the resin and simultaneously deprotected by treatment with cleavage cocktail (TFA:EDT:EMS:anisole = 93:1:3:3) for 4 h. After cleavage, the peptide-resin mixtures were filtered, precipitated with ice-cold diethylether, and pelleted by centrifugation. Lyophilized peptides were purified by RP-HPLC using a C18 column (PMC Pack-488AM, 10 $\times$ 150 mm). HPLC gradients were run over the range 20–50% B buffer at 3 mL/min over 30 min (A buffer: 0.1% TFA in water, B buffer: HPLC-grade acetonitrile). Peptides cyG7A5 and cyG7A5C were intramolecular-disulfide cyclized by adding the peptide solution dropwise into 30 mM Tris-HCl (pH 8.5) and stirring overnight. The N-terminal Thz in cyG7A5C was converted to Cys by adding 0.2 M methoxyamine-HCl (pH <4) and incubating for 2 days. Purified peptides were characterized by MALDI-TOF-MS (cyG7A5: calc. 4624.166; observed 4623.352 [M + H], cyG7A5C: calc. 4784.196; observed 4783.99 [M + H], linG7A5C: calc. 4649.215; observed 4650.593 [M + H]).

4.3. Surface plasmon resonance

The SPR experiments were conducted on a Biacore T200 instrument (GE Healthcare). Peptide cyG7A5C was immobilized onto a CM5 sensor chip using a thiol-coupling procedure. Blank surfaces were obtained in the same manner without injection of peptides. Experiments were conducted in HBS-EP⁺ buffer with 1% BSA (GE Healthcare, 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.1% [v/v] surfactant P20, pH 7.4) at 25°C at a flow rate of 30 mL/min. Human IgG-Fc, human full-length IgG, HSA, fibrinogen, and BSA were also analyzed.

4.4. Protease stability

Peptides linG7A5C and cyG7A5C were dissolved in Tris-HCl buffer (pH 8.5) to a final concentration of 42 μ M. Trypsin was dissolved in Tris-HCl buffer (100 mM, containing 1% glycerol, pH 8.5) to a final concentration of 240 μ g/mL. Next, a 50 μ L volume of each peptide solution was incubated with trypsin at 37°C. Digestion of peptides by trypsin was monitored by RP-HPLC at different times to determine the fraction of protease degradation.

4.5. CD spectra

CD spectra were recorded on a Jasco J-820 spectropolarimeter (Jasco) equipped with a Pelitier PTC-423L thermo-unit (Jasco). Far-UV CD spectra (260-190 nm) were recorded using a 0.1-cm path length cell under constant nitrogen flush with a step size of 0.2 nm, bandwidth of 1 nm, and averaging time of 2 seconds at 20°C. The final spectra reported were an average of 5 scans.

4.6. Immobilization of IgG-Fc binding peptides

PVAc resin (18 g) was reacted with epichlorohydrin (1.78 mol), sodium hydrate (0.53 mol), and sodium borohydride (2.33 mmol) in DMSO for 5 h at 30°C. After filtration, the swelling resin (30 mL) was reacted in 28 % aqueous ammonia (45 mL) for 1.5 h at 40°C. The aminated PVAc resin (5.0 mL) was reacted with succinimidyl-[(N-maleimidopropionamido)-diethyleneglycol] ester (0.1 mmol) in DMSO (10 mL) for 3 h at 30°C. The maleimide-activated resin (1.5 mL) was conjugated with peptide (2.4 mM) in PBS solution for 17 h at 37°C. Peptides cyG7A5C and linG7A5C were immobilized at 3.8 µmol/mL and 1.8 µmol/mL of gel, respectively. Protein A was reacted with epoxidized PVAc resin and immobilized at 0.18 µmol/mL of gel.

4.7. Batch-wise protein adsorption

Plasma for this study was obtained by centrifugal separation from two healthy donors. Plasma (3 mL) was mixed with each IgG-binding ligand immobilized on the PVAc gel. Each batch-wise adsorption test (volume ratio of gel to plasma = 1:6) was carried out by shaking for 17.5 min at 37°C, after which the IgG-binding ligand immobilized on the PVAc gel was removed by centrifugation. Percent protein removal was calculated using the following equation:

$$\% \text{ removal} = 100 \cdot (C_0 - C) / C_0,$$

where % removal is the ratio of each protein adsorbed onto the IgG-binding ligand immobilized on the PVAc gel, C_0 represents the concentration of protein in the initial plasma solution, and C represents the concentration in the plasma supernatant after batch-wise adsorption. The concentration of human IgG was determined by immune nephelometry, and the concentration of fibrinogen was determined by clotting time measurement.

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