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Letter

# New discovery of high-affinity SARS-CoV-2 spike S2 protein binding peptide selected by PhIP-Seq

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*Dear Editor,*

The world had suffered pandemics of COVID-19 caused by SARS-CoV-2. At the time of writing, COVID-19 is still widely spreading in many countries and regions, and by June 14, 2022, totally 532,887,351 cases, including 6,307,021 deaths, were reported globally (WHO, https://covid19.who.int/table). Powerful measures, such as prophylactic and therapeutic interventions against the virus, are in urgent need for COVID-19 control and prevention.

The spike protein, which is expressed on the virus surface, is the key determinant of viral-host interaction (Ng et al., 2021) and mediates the virus entry, as the receptor-binding domain (RBD) of spike protein is responsible for binding to host receptor and the S2 subunit is responsible for membrane fusion (Harvey et al., 2021). Thus, interventions targeting SARS-CoV-2 spike protein are quite effective to fight against the virus. Spike protein binders, such as antibodies, miniproteins, peptide binders and DNA binders, have protective potential against SARS-CoV-2 infection (Gavor et al., 2020; Case et al., 2021; Singhal et al., 2021). With good accessibility, easy handling, easy modification, low immunogenicity, and low steric effect, peptide binders have been focused on. Peptides binding to spike protein with high affinity and specificity can contribute to developing diagnostic measures, therapeutic options, and learning virus entry (Ucar et al., 2021; Pomplun et al., 2021). However, at present, few peptides majorly targeting RBD had been identified by rational design, repurposing of anti-viral peptides, and selection (Zhao et al., 2020; Norman et al., 2021; Pei et al., 2021).

In this study, we selected peptide binders for SARS-CoV-2 spike trimer protein by using phage immunoprecipitation-sequencing (PhIP-Seq) technology with a phage-displayed random peptide library. Phage selection was usually based on panning technology combined with Sanger sequencing. However, useless results were often obtained since phages can not only bind to the target but also the nontarget subjects (including the container walls, the carriers, and irrelevant molecules), and target-unrelated phages can amplify to a large amount along with the panning progress. To decrease non-specific adsorption of phages and obtain more valuable information on protein-peptide interactions, we established a four-round alternant panning method combining amplicons next-generation sequencing (NGS) technology to select peptide binders for spike protein (Supplementary Fig. S1). As a huge amount of data would be produced by NGS, a "One-click" operation system running on MATLAB software has been developed to simplify the data processing. After normalizing the peptide reads into standardized scores, we compared scores between spike protein and negative control (NC), and 50 peptides were identified. After four rounds of selection, the peptide NFWISPKLAFALGGGS (named Spep-1) showed significant enrichment (Supplementary Fig. S2). MEME Discovery of MEME suite (https://meme-suite.org/meme/tools/meme) was used to analyze the conserved motifs, and we found that most peptides appeared to share a similar motif with NFWISPKLAFALGGGS. Interestingly, Spep-1 peptide showed the identical sequence with the motif (Fig. 1A).

Seven peptides were chosen and synthesized, and their binding ability to spike trimer protein was evaluated by peptide-capture ELISA assay (Supplementary file). Results showed that Spep-1 peptide had an excellent binding ability to spike trimer protein (Supplementary Fig. S3). Peptide-capture ELISA assay was conducted to determine the binding domain of Spep-1 on spike protein. RBD proteins, S1 protein, S2 protein, the ectodomain of spike trimer, and monomer proteins were incubated

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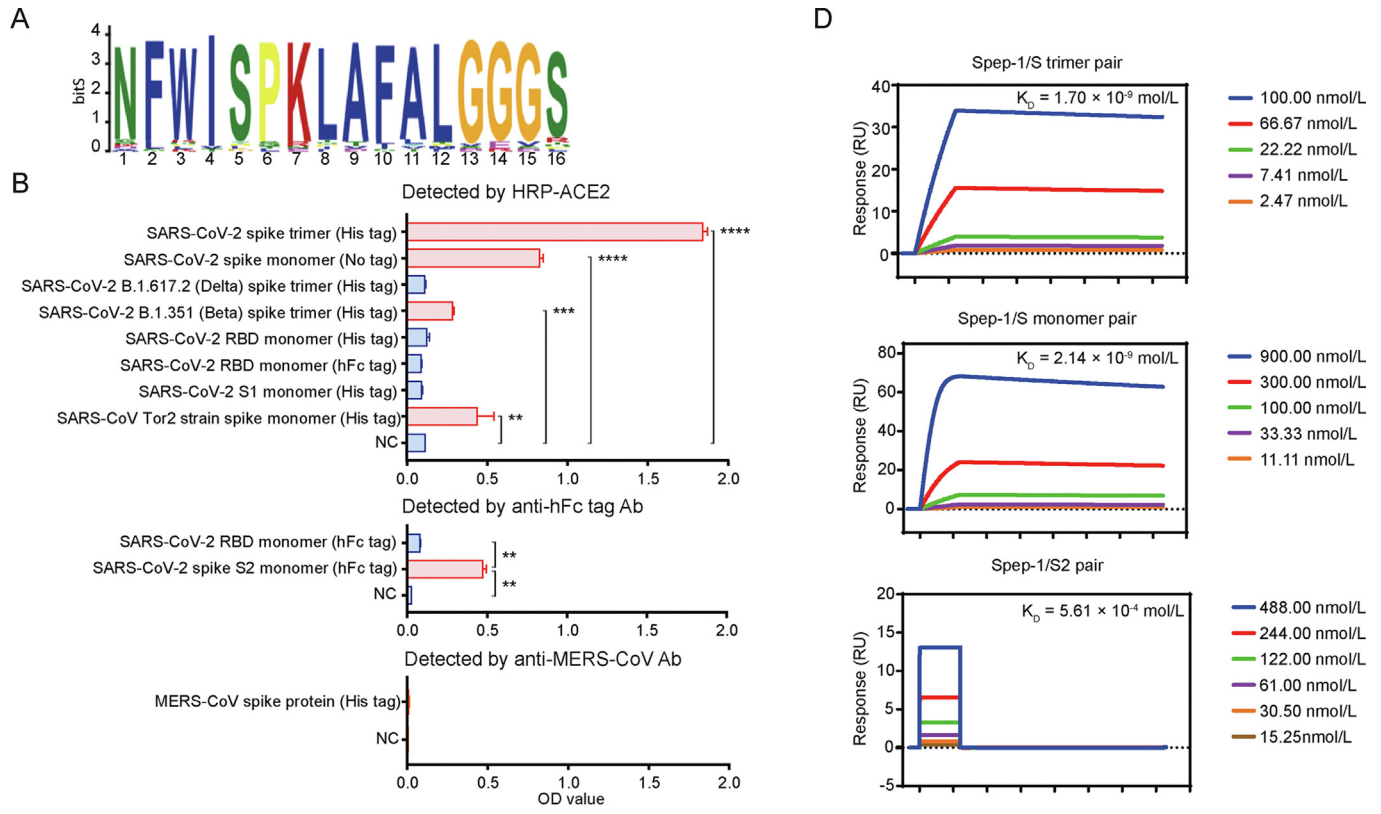


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**Fig. 1.** The high-affinity peptide binder Spep-1 for SARS-CoV-2 spike protein. **A** Motif of the screened peptides identified by MEME. **B** The binding activity of Spep-1 to spike proteins and subunits of SARS-CoV-2, SARS-CoV, and MERS-CoV. **C** The detailed interactions between Spep-1 and SARS-CoV-2 spike trimer protein (ectodomain). The key amino acids included Ile788, Lys790, Thr791, Lys795, Pro809, Lys814, Gln872 and Glu702. S2 monomers are shown in green and blue respectively. Adjacent S1 monomers are shown in red and Spep-1 is shown in yellow. **D** The binding kinetics between Spep-1 peptide/SARS-CoV-2 spike trimer protein pair, Spep-1 peptide/SARS-CoV-2 spike monomer protein pair, and Spep-1 peptide/SARS-CoV-2 spike S2 protein pair. The former of the pair was used as the ligand in SPR assay while the latter as the analyte. **E** SARS-CoV-2 spike trimer protein can be captured by Spep-1 peptide from human serum matrixes. Left panel: SDS-PAGE assay stained by Coomassie brilliant blue. Right panel: Western blot (WB) assay detected by anti-RBD mAb 34B4. Pull-down 1 and 2: spike trimer protein pull-down from SD-1 by Spep-1-bio and Control-bio respectively; 3 and 4: spike trimer protein pull-down from human serum B1 by Spep-1-bio and Control-bio respectively; 5 and 6: spike trimer protein pull-down from human serum B3 by Spep-1-bio and Control-bio respectively. **F** Sandwich ELISA assays for detection of SARS-CoV-2 spike proteins in spiked serum samples based on peptide capture/mAb detection systems. The blue dotted lines indicate the lower limit of detection (LLOD) of the Peptide-capture/24G1-detection system, green for the Peptide-capture/24G2-detection system, and red for the Peptide-capture/24C7-detection system. **G** Sandwich ELISA assay based on peptide-capture/24C7-detection system for detection of SARS-CoV-2 pseudovirus.

with Spep-1 respectively and the binding complexes were detected by different antibodies (Supplementary file). The results showed that Spep-1 has a binding ability with spike trimer, monomer protein and S2 subunit (Fig. 1B). Further study showed that Spep-1 can also bind to spike protein of SARS-CoV-2 beta variant and SARS-CoV, but shows no binding activity to spike protein of SARS-CoV-2 delta variant and MERS-CoV.

To further study Spep-1's specific target on S2 subunit, molecular docking analysis was also performed. In the docked complex, 18 amino acids of S2 subunit (Ile788, Tyr789, Lys790, Thr791, Pro792, Pro793, Ile794, Lys795, Ser803, Leu806, Pro807, Asp808, Pro809, Ser813, Lys814, Gln872, Ser875 and Glu702) mediated interactions with Spep-1 peptide (Fig. 1C). Mutation energy analysis showed that Ile788, Lys790, Thr791, Lys795, Pro809, Lys814, Gln872 and Glu702 were the key amino acids (Supplementary Fig. S4).

To measure the affinity between Spep-1 peptide and SARS-CoV-2 spike proteins, we performed surface plasmon response (SPR) assays by using Biacore kinetic assays. Two analyses were conducted: in the first method, Spep-1 peptide was used as the analyte, while spike proteins were used as the analyte in the second method (Supplementary file). The Biacore kinetic assays showed that Spep-1 peptide bound to spike trimer protein and monomer protein with dissociation equilibrium constants $K_D$ of $9.28 \times 10^{-8}$ mol/L and $1.18 \times 10^{-6}$ mol/L respectively in the former assay (Supplementary Figs. S5A and S5B). In the latter assay, spike trimer and monomer protein bound to Spep-1 peptide with $K_D$ value of $1.70 \times 10^{-9}$ mol/L and $2.14 \times 10^{-9}$ mol/L respectively (Fig. 1D). S2 monomer protein also showed binding activity to Spep-1, however, the $K_D$ value only reached $5.61 \times 10^{-4}$ mol/L (Fig. 1D). This may be due to the conformational change of S2 protein we used.

Since spike proteins are bound to the immobilized Spep-1 peptide with high affinity, we further explored the potential usage of Spep-1. Spike trimer protein was mixed with SD-1 buffer and human serum collected from healthy donors to mimic the serum from the infected patient. Then the mixtures were incubated with biotin-labeled Spep-1 peptide or control peptide immobilized on SA beads. The captured proteins were analyzed by Western blotting using anti-SARS-CoV-2 spike RBD protein murine monoclonal antibody (Supplementary file). The results showed that Spep-1 could enrich spike trimer protein from human serum with high yields, while the control peptide had low yields in different matrixes (Fig. 1E). Those results proved that Spep-1 could be used in the detection of SARS-CoV-2 spike protein in serum samples.

The SPR assay showed that the dissociation rate of spike protein was extremely slow when Spep-1 was immobilized as the ligand and this prompted that Spep-1 can be used as an excellent capture molecule for spike antigen. Moreover, the binding of Spep-1 to spike protein caused little steric hindrance effect. Thus, sandwich ELISA assays based on Spep-1 capture/mAb detection system can be easily established and may have good performances. To verify the scheme, biotin-labeled Spep-1 or control peptides immobilized on SA-coated plate was used as capture molecules for spike trimer or monomer protein, and three HRP conjugated mAbs 24C7, 24G1 and 24G2 that recognize different epitopes were randomly selected as detection antibodies. Buffers for diluting spiked serum samples and HRP-conjugated mAbs were screened, and the best detection performance was conducted by using ED13 buffer for diluting both (Supplementary Fig. S6). Among the three detection systems, Spep-1/24C7 worked best and reached the detection limit of 0.76 pmol/L for both spike trimer protein and monomer protein in serum samples. The detection limit of 24G1 reached 6.96 pmol/L for spike trimer protein and 20.58 pmol/L for monomer protein, while the detection limit of 24G2 was 6.86 pmol/L for spike trimer protein and 0.76 pmol/L for monomer protein (Fig. 1F). Other studies also established immunoassays based on peptide binders, a high-affinity peptide binding to RBD of spike protein was selected by synthetic peptides-mass spectrometry technology which reached a lower limit of detection (LLOD) of 100 pmol/L spike RBD protein in spiked serum samples (Pomplun et al., 2021), and another RBD-binding cyclic peptide discovered by mRNA display technology reached an LLOD of 31.25 ng/mL (equivalent of 78.13 pmol/L) spike trimer protein (Norman et al., 2021). Compared with those researches, Spep-1 immunoassay increased the sensitivity by more than 100 times. Besides spike protein, the system can also detect SARS-CoV-2 pseudovirus in contrived serum samples (Fig. 1G).

In this study, by using the optimized PhIP-Seq methodology, we newly selected a peptide binding to the S2 subunit of SARS-CoV-2 spike protein and investigated the potential application of this peptide in clinical diagnostics. Since S2 subunit performs an important role in viral infusion and entry, it is also more conserved to be the target site for anti-viral inhibitor development (Ng et al., 2021). Our data showed that Spep-1 had a high affinity to SARS-CoV-2 spike trimer and monomer protein, with the $K_D$ values of 1.70 and 2.14 nmol/L (Spep-1 as the ligand in SPR analysis), which are quite high among the reported peptides (Mendoza-Figueroa et al., 2018; Hao et al., 2019; Pomplun et al., 2021; Rosestedt et al., 2021), suggesting that Spep-1 may have good application prospects, not only limited to SARS-CoV-2 spike antigen detection. In future studies, with rational modifications, Spep-1 might be used for studying the spatial and temporal distribution of S2 subunit during virus infusion, *in vivo* virus tracing, and drug delivery in biosafety laboratories. Although Spep-1 without modification didn't show neutralizing activity based on SARS-CoV-2 pseudovirus neutralization assay established in our previous report (Xiong et al., 2020) (Supplementary Fig. S7), the data obtained in the current study proved that our PhIP-Seq platform could be used to select functional peptides. And we plan to select peptides with neutralizing activity against the RBD region of SARS-CoV-2 spike protein in the next step.

## Footnotes

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All the data generated during the current study are included in the manuscript and supplementary material. Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2022.07.001.

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