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Selection of ovalbumin-specific binding peptides through instant translation in ribosome display using *E. coli* extract

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Abstract

In vitro selection has been widely used to generate molecular recognition elements in analytical sciences. Although reconstituted types of in vitro transcription and translation (IVTT) system, such as PURE system, are nowadays widely used for ribosome display and mRNA/cDNA display, utilizing *E.coli* extract tends to be avoided presumably because it contains unfavorable contaminants such as ribonuclease. Nevertheless, the initial speed of protein translation in *E.coli* extract is markedly faster than that of PURE system. Thus, we hypothesized that *E.coli* extract is more appropriate for instant translation in ribosome display than PURE system. Here, we first revisit the potency of *E. coli* extract for ribosome display by shortening the translation time and then applied the optimized condition to the selection of peptide aptamers for ovalbumin (OVA). The OVA-binding peptides selected using *E.coli* extract exhibited specific binding to OVA even in the presence of 50% serum. We conclude that instant translation in ribosome display using *E.coli* extract has the potential to be used to generate easy-to-use and economical molecular recognition elements in analytical sciences.

**Keywords:** Aptamer, Food allergen, in vitro selection, Dot blot
**Introduction**

In vitro selected aptamers have been recently used as molecular recognition elements in place of antibodies in analytical sciences.\(^1\)\(^-\)\(^3\) Ever since various in vitro selection techniques to obtain aptamers have been explored after phage display system was developed by Smith in 1985,\(^4\) aptamers are increasingly becoming prevalent. In the phage display system and similar systems involving cells at any in vitro selection steps, the genetic diversity of displayed proteins/peptides is largely suppressed because the number of transformable DNA is far less than the measure of genetic diversity.\(^5\), \(^6\) Such loss of genetic diversity can be avoided by utilizing ribosome display, mRNA/cDNA display and others that campaign in vitro selection using in vitro transcription and translation (IVTT) system.\(^7\)\(^-\)\(^10\)

While the first demonstration of ribosome display used *E.coli* cell extract for IVTT,\(^11\) reconstituted type of IVTT systems are employed nowadays since Shimizu et al. reported reconstituted IVTT system (PURE system) by combining recombinant protein factors with purified 70S ribosomes.\(^12\) For example, reconstituted IVTT systems including PURE system, PUREflex\(^®\) and PURExpress\(^®\) are widely used for ribosome display and mRNA/cDNA display than *E.coli* extract, presumably because such systems are highly modifiable and *E.coli* extract contains unfavorable contaminants such as ribonuclease. Nevertheless, Villemagne et al. reported that the initial speed of protein translation in *E.coli* extract is markedly faster than that of PURE system.\(^13\) We hypothesized that utilizing *E.coli* extract can shorten the translation time in the selection process, which can suppress the RNA degradation. Thus motivated, we first revisited the potency of *E. coli* extract for ribosome display by shortening the translation time and then applied this optimized condition to the selection of peptide aptamers for ovalbumin (OVA) which is a well-known allergen in egg white.
Experimental

Reagents and oligonucleotides

All reagents were of extra pure grade and were used as received without further purification. All primers and template DNAs were purchased from Eurofins Genomics (Japan).

Demonstration of in vitro selection using E.coli extract

First, to demonstrate in vitro selection using anti-flag antibody modified magnetic beads, two template sequences: a positive sequence containing FLAG tag (FLAG, 657 bp) and a negative junk sequence not containing FLAG (JUNK, 855 bp) were prepared (Fig. 1A). Both DNA templates contain the genetic components which were required for ribosome display; T7 promoter (T7p) and Shine-Dalgarno (SD) sequence at the upstream of the ORFs, a helical linker and the ribosome arrest sequence (SecM) at the C-terminus of the ORFs\textsuperscript{14}. Each DNA template was transcribed using the MEGAscript\textsuperscript{TM} T7 transcription kit (Thermo fisher scientific, MA, USA) and subsequently subjected to TURBO DNase treatment. The transcribed mRNAs were purified using the mRNA clean and concentrator kit (Zymo Research, Irvine, CA, USA). Purified JUNK and FLAG mRNAs were mixed together at 9 : 1 ratio and used to demonstrate ribosome display using E.coli extract.

The mRNA mix (5 μg) was mixed with preheated E.coli extract at 37 °C (final 25 μL). E.coli strain (B-95.ΔA) and RF-1 deficient strain\textsuperscript{15} was used for preparation of E.coli extract by following the previously reported method by Kigawa et al.\textsuperscript{16} After 5, 10 or 15 minutes of translation at 37 °C, 200 μL of ice-cold WBT buffer (50 mM Tris/acetate, 150 mM NaCl, 50 mM magnesium acetate, 0.05% Tween 20, pH 7) and
anti-FLAG antibody immobilized magnetic beads (MBL, Japan) were added, which were pre-washed three times with WBT buffer. The mixture was gently shaken for 30 minutes at 4 °C. Next, the anti-FLAG antibody immobilized beads were washed eight times with ice-cold WBT buffer to remove any unbound complexes. mRNA bound to the beads were eluted using heat at 70 °C for 5 minutes in the elution buffer (7M urea in WBT buffer). After purification of the mRNA using the mRNA clean and concentrator kits, the mRNA was reverse-transcribed to cDNA (SuperScript® IV, Thermo fisher scientific). cDNA was amplified using PrimeSTAR GXL DNA polymerase (Takara Bio, Japan) and with the primers (5’-CGAAATTAATACGACTCATAAGGAGACCACAACGTTTC-3’ and 5’-TTAGCTCACCAGAAATACATCTG-3’). As control, input mRNA was diluted 1000 folds and subjected the reverse-transcription and the subsequence processes. Finally, enrichment of the FLAG sequence through the selection was analyzed using poly-acrylamide gel electrophoresis. The image of poly-acrylamide gel was recorded using a gel imager (Luminograph II, ATTO, Japan). The band intensity was analyzed by using Igor Pro (WaveMatrix, USA).

Preparation of random library for OVA-binding aptamers

For the selection of OVA-binding peptides (OvaBPs), previously reported protocol14 was executed. As an exception, the following two single-stranded DNA libraries:

5’-TGCGTCCGTCTCCTATGACATAAGGCGGTCTGGCAGCGGCGAGC
GGTCAGCTTCGTAACATGT(10VN)TGTTCTTGGGACAAGAGAC
GGTCAGC-3’ and
5’-TGCGTCCGTCTCCTATGACATAAGGCGGTCTGGCAGCGGCGAGC
GGTCAGCTTCGTAACATGT(10NNK)TGTTCTTGGGACAAGAGAC
GGTCAGC-3’, where V is A, G, or C, N is A, T, G, or C, K is G or T, were used for this
study. The prepared DNA templates were transcribed using the MEGAscript™ T7 transcription kit and subsequently subjected to TURBO DNase treatment. Finally, the transcribed mRNAs were purified using the mRNA clean and concentrator kit. Purified VVN and NNK mRNAs were mixed together at 9 : 1 ratio and used for the selection of OvaBPs using *E.coli* extract.

**Selection of OvaBPs**

The RNA mix (10 μg) was mixed with *E.coli* extract in which 2μL of RNase OUT® (Thermo fisher scientific) was present (total 50 μL). After 5 minutes incubation at 37 °C for translation, 200 μL of ice-cold WBT buffer (50 mM Tris/acetate, 150 mM NaCl, 50 mM magnesium acetate, 0.05% Tween 20, pH 7) and OVA (albumin from chicken egg white lyophilized powder, Sigma-Aldrich, Germany) attached to magnetic beads (Dynabeads™ M-270 carboxylic acid, Thermo Fisher Scientific), which were pre-washed three times with the WBT buffer, were added. The mixture was gently shaken for 30 minutes at 4 °C. The OVA-immobilized beads were washed eight times with ice-cold WBT buffer to remove any unbound complexes. mRNA bound to the beads were eluted using heat at 70 °C for 5 minutes in the elution buffer (7M urea in WBT buffer). After purification of the mRNA using the mRNA clean and concentrator kits, the mRNA was reverse-transcribed to cDNA using SuperScript® IV. cDNA was amplified using PrimeSTAR GXL DNA polymerase and primers (5’-CGAAATTAATACGACTCAGAGGAGCCACACGTTTC-3’ and 5’-TTAGCTCACCAGAATATCATCTG-3’). After purification using Nucaway®, the PCR product was used as a template for the next round of selection. To obtain OVA-specific peptide aptamers, we performed a more stringent selection in the latter rounds of selection which includes a negative selection using ovomucoid-modified
magnetic beads. (Table S1). At the end of each round of selection, we sequenced the enriched DNA library using next generation sequencing (NGS).

**Peptide Synthesis**

10 OVA-binding peptides (OvaBPs) were synthesized using automated microwave peptide synthesizer (Liberty Blue, CEM, Japan) by following manufacturer’s protocol. Briefly, reagents include; 0.05 mmol resin (Fmoc Rink Amide ProTide Resin, CEM), N,N-dimethylformamide (DMF) as the main solvent, 20% piperidine in DMF for deprotection, 0.2M Fmoc-amino acids in DMF, 0.5M N,N'-Diisopropylcarbodiimide (DIC) in DMF as the activator and 0.5 M Oxyma in DMF as the base. Fmoc-amino acids and Oxyma were purchased from CEM and DMF, piperidine, DIC were purchased from Fujifilm Wako Chemicals (Japan).

To modify fluorescein (FAM) to the N-terminus of the synthesized peptides, peptides on the resins were mixed with 0.02 mM NHS-activated FAM (Thermo fisher scientific) and 0.5 M Oxyma in DMF. After incubation at 50 °C for 1 hour, the resins were washed with methanol and the resins were collected.

To cleave the peptides from the resins, the resins were incubated in the cleavage cocktail (5 μL of phenol, 5 μL of H2O, 5 μL of Thioanisole, 2.5 μL of 1,2-Ethanediethiol, 1 μL of Triisopropylsilane, 81.5 μL of Trifluoroacetic Acid) at 40 °C for 1 hour. After filtering the solution to remove the resins, the filtered solution was mixed with pre-cooled diethyl ether (-20 °C). After centrifuging the solution for 15 minutes to precipitate the peptide, the supernatant was removed. The precipitated peptide was washed using pre-cooled diethyl ether 4 times and dried in a hood.

Each peptide was further purified using a high-performance liquid chromatograph (Extrema, JASCO, Japan) equipped with a C18 column (COSMOSIL C18-AR-II,
A mixture of solvent A (0.1% TFA in water) and B (0.1% TFA in acetonitrile) was used as the mobile phase; and a linear gradient from 5% to 60% B for 1 hour at a flow rate of 4.0 mL/min was applied. The masses of the eight purified peptides were verified by MALDI-TOF-MS (microflex LT, Bruker Daltonics, USA); OvaBP1, m/z = 2458.766 [M+H]+, calc. m/z = 2458.116; OvaBP2, m/z = 2349.509 [M+H]+, calc. m/z = 2349.132; OvaBP3, m/z = 2123.728 [M+H]+, calc. m/z = 2122.897; OvaBP4, m/z = 1999.597 [M+H]+, calc. m/z = 1999.89; OvaBP6, m/z = 2357.595 [M+H]+, calc. m/z = 2357.097; OvaBP8, m/z = 2339.240 [M+H]+, calc. m/z = 2339.122; OvaBP9, m/z = 2274.370 [M+H]+, calc. m/z = 2273.109; OvaBP10, m/z = 2419.893 [M+H]+, calc. m/z = 2419.072, FAM-OvaBP1, m/z = 2817.472 [M+H]+, calc. m/z = 2816.164; FAM-OvaBP2, m/z = 2708.531 [M+H]+, calc. m/z = 2707.18; FAM-OvaBP3, m/z = 2482.056 [M+H]+, calc. m/z = 2480.945; FAM-OvaBP4, m/z = 2359.786 [M+H]+, calc. m/z = 2357.938; FAM-OvaBP6, m/z = 2716.635 [M+H]+, calc. m/z = 2715.145; FAM-OvaBP8, m/z = 2699.838 [M+H]+, calc. m/z = 2697.17; FAM-OvaBP9, m/z = 2633.210 [M+H]+, calc. m/z = 2631.157; FAM-OvaBP10, m/z = 2777.096 [M+H]+, calc. m/z = 2777.12 (monoisotopic mass for all). To note, OvaBP5 and OvaBP7 were not successfully synthesized, and thus were unavailable for further experiments.

**Dot blot assay**

First, stock solutions of OVA and negative control protein, bovine serum albumin (BSA), were prepared at 5 mg/mL and series of two-fold dilution of each stock solution was performed to prepare 7 different concentrations for each protein. The PVDF membrane (Immobilon-FL PVDF Membrane, Merck Millipore, USA) was immersed in methanol for 30 minutes for hydration. The hydrated PVDF membrane was transferred
to the transfer buffer (25 mM tris, 192 mM glycine, 20% methanol, 80% H2O) and incubated for 5 minutes with gentle shake. The PVDF membrane was laid over Whatman® filter papers which were pre-immersed in the transfer buffer. After casting 2 μL of each concentration of OVA and BSA solutions on the PVDF membrane, the membrane was dried to immobilize the protein in the PVDF membrane. The dried PVDF membrane was hydrated again by immersing in methanol and transfer buffer with gentle shake for a minute each. The membrane was transferred into the blocking solution [0.75 g of protein blocking agent (ECL prime™ blocking agent, Cytiva, Japan) and 14.25 mL of TBST (Tris-buffered saline with Tween 20, 0.05 mM Tris-HCl, 0.15 M NaCl, 0.05% Tween™ 20) for overnight with gentle shake. Then, the PVDF membrane was rinsed three times with TBST for 5 minutes each. The membrane was transferred into the solution containing each FAM-modified OvaBPs (0.3 μM FAM-OvaBPs in TBST) for staining the protein spots for 1 hour in dark. The membrane was rinsed with TBST to wash off unbound OvaBPs. The fluorescence image of PVDF membrane was visualized by excitation at 505 +/- 25 nm (CyanoView, ATTO, Japan) and recorded using Luminograph II.

Another dot blot analysis was performed to demonstrate the specificity of OvaBPs even in the presence of serum. Solutions containing a series of concentration of serum (fetal bovine serum, Biosera, Nuaille, France), 50%, 25%, 10%, 5% and 0% in the presence and absence of 5 mg/mL OVA were prepared. The dot blot assay was performed as mentioned above using these solutions instead of OVA and BSA.

**Determination of dissociation constants**

The dissociation constants ($K_d$) of OvaBPs were determined using BLItz (Pall ForteBio, USA). Biotinylated OvaBPs were prepared by mixing 5 μL of 250 μM
OvaBPs with 5 μL of 2.5 mM EZ-Link™ Maleimide-PEG2-Biotin (Thermo fisher scientific) and incubated overnight at room temperature. For OvaBP9, which does not possess Cys in the sequence, 10 μL of 10mM EZ-Link™ Sulfo-NHS-LC-Biotin (Thermo fisher scientific) was used in place of Maleimide-PEG2-Biotin. The biotinylated OvaBPs were purified using HPLC as mentioned above in the section of the purification of OvaBPs and FAM-OvaBPs.

BLI buffer (Phosphate buffered salts, 0.002% Tween 20) was used for all solutions used in BLItz. 0.25 μM biotinylated OvaBPs were loaded to streptavidin coated biosensors. Three concentrations of OVA, 10, 5 and 2.5 μM, were used for the association step. For biotinylated OvaBP9, 50 and 10 μM OVA were used for the association step. The dissociation constants between OVA an OvaBPs were determined by the BLItz software.

Results and Discussion

Demonstration of ribosome display using E.coli extract

To revisit the utilization of E.coli extract in ribosome display, we first constructed a test system. In this system, we performed in vitro selection from a mixture of DNA sequence containing FLAG (DYKDDDK in amino acid) and JUNK sequence at 1:9 ratio and enriched the FLAG sequence via the selection with anti-FLAG antibody immobilized beads (i.e. the JUNK sequence should not be captured by the anti-FLAG antibody immobilized beads whereas the FLAG sequence should be captured and enriched through the selection). Because the length of the JUNK sequence, 855 bp, is longer than that of FLAG sequence, 657 bp, the enrichment of FLAG sequence can be monitored via gel electrophoresis after the amplification using PCR.

Using this test system, we explored translation time to demonstrate that E.coli
extract is appropriate for instant translation in ribosome display. Since Villemagne et al. reported that *E. coli* extract can produce a single chain antibody fragment within seven minutes\textsuperscript{13} we investigated the enrichment of FLAG sequence in 5, 10, 15 minutes of translation (Fig. 1B). As expected, the control (e.g. sample without selection) exhibited strong JUNK band whereas the FLAG sequence was enriched for all three translation times. To note, we found that even five minutes of translation was sufficient to enrich FLAG sequence with marginal recovery of JUNK sequence and enrichment efficiencies obtained from each translation times were not significantly different. We also found that longer translation may cause recovery loss of the FLAG sequence. For example, the intensity of the FLAG band after 25 PCR cycles (15 minutes translation), 375,000 a.u., was comparable with that in fewer 20 PCR cycles (5 minutes translation) which was 320,000 a.u.. This indicates that FLAG sequence recovered in 15 minutes translation was less than the amount recovered from 5 minutes translation. Perhaps, this recovery loss arises from ribonuclease activity in *E. coli* extract. We also compared the enrichment efficiency of FLAG sequence between *E. coli* extract and a reconstituted translation system (PURE system) using a similar test system including an unnatural amino acid and found that *E. coli* extract can effectively enrich FLAG sequence than PURE system (Fig. S1). Overall, these results clearly indicated the potency of *E. coli* extract for ribosome display by shortening translation time.

*Selection and preparation of OvaBPs*

Next, we applied the five minutes translation in ribosome display system using *E. coli* extract for selecting OvaBPs. OVA is the most abundant protein in egg white,\textsuperscript{17} chicken egg allergy is the second most common food allergy (first is cow’s milk allergy).\textsuperscript{18} The detection of OVA in food is important to avoid serious reactions of the immune system,
We first prepared two types of random-sequence DNA library containing ten continuous NNKs and VVNs (N represents A, T, G, or C, K represents G or T, and V represents A, G, or C). The NNK codons cover 32 codons out of 64 codons and are translated into the 20 canonical amino acids and TAG stop codon (Fig. 2B); The VVN codons covers 36 codons out of 64 codons and are translated into 12 canonical amino acids (Fig. 2C). Because the 12 amino acids which were translated from VVN codons were mostly hydrophilic or neutral amino acids (as much as nine amino acids out of the 12 amino acids), the expected value of hydrophobic amino acids per one peptide in the 10 continuous VVN codons was 3.3, lower than that for NNK codons, 4.7. Considering the difficulties in synthesizing highly hydrophobic peptides (typically over 50% in amino acids) using solid-phase peptide synthesis, the mRNA libraries which were transcribed from the VVN and NNK libraries were mixed together at the ratio of 9:1 (Fig. 2D).

Next, we campaigned the five minutes translation in ribosome display against OVA. In this selection, OVA-immobilized magnetic bead selection was used for positive selection, while naked magnetic beads and ovomucoid-immobilized beads were used for negative selection (Table S1). After the eight rounds of selection, the DNA sequences obtained after each round of selection were sequenced using next generation sequencing. Among the obtained 74,392 reads (6,424 unique sequences) at the end of the eighth round, we found that 26% of the reads were derived from the VVN library even though we started the selection from the library containing theoretically over 90% (VVN)10 sequences (Fig. 2E). This trend in selection is potentially useful to design another library for ribosome display using E.coli extract.

We targeted synthesis of top ten peptides which were enriched during sixth to eighth rounds which included the negative selection using ovomucoid (Table 1); however, we...
failed to synthesize OvaBP5 and OvaBP7 presumably due to deficiency in purification of cleaved peptides from resin beads. For the other eight peptides, we also prepared biotinylated and fluorescence-modified cognate peptides for kinetics measurement and dot blot assay, respectively.

*Kinetic binding measurement*

We confirmed the binding of OvaBPs to OVA using bio-layer interferometry (BLI). In this experiment, we first immobilized each biotinylated-OvaBP on streptavidin-coated biosensor and monitored the association and dissociation processes of OVA with the OvaBP immobilized biosensor (Fig. S2 and S3). We found that all eight OvaBPs exhibited OVA binding; however, OvaBP3 exhibited monophasic association with OVA while the rest of OvaBPs exhibited multiphasic association. Because non-specific binding of OVA to the streptavidin-coated biosensor was not observed (Fig. S2), we hypothesized two possibilities behind multiphasic binding; multi-form of the immobilized OvaBPs and dimerization of OVA. Although two Cys residues in each OvaBPs except for OvaBP9 were potentially biotinylated (OvaBP9 does not possess Cys in the sequence.), single- and double-biotinylated OvaBPs were not completely separated in HPLC purification. Another possibility of multiphasic binding could arise from OVA conformation; reports from Ianeselli et al. and Nemoto et al. suggest that OVA can form a dimer.21, 22 Thus, OvaBPs may bind to both monomer and dimer forms of OVA or only either forms, which may result in the multiphasic behavior. Although no attempt has been made to clarify the origin of the multiphasic behavior, we confirmed the binding of the eight OvaBPs to OVA using BLI.
**Dot blot assay**

To examine whether the selected OvaBPs specifically bind OVA, we performed dot blot assay with OVA, ovomucoid (OVM) and bovine serum albumin (BSA) (Fig. 3A, S4 and S5). Here, most OvaBPs exhibited OVA concentration dependence of the fluorescence signal while some OvaBPs exhibited high background signal (Fig. S5). None showed binding to OVM and BSA. Next, we investigated whether the selected OvaBPs can bind to OVA in the presence of serum (Fig. 4). Even in the presence of 50% serum, some OvaBPs exhibited binding to OVA. Since *E. coli* extract contains various biomaterials including proteins and oligonucleotides, the aptamer selected from such contaminated extract may be robust. Furthermore, short translation times could suppress RNA degradation by RNase present in *E. coli* extract. **Taken together, we conclude that instant translation in ribosome display using *E. coli* extract has the potential to be used to generate easy-to-use and economical molecular recognition elements in analytical sciences.**

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### Table 1 Ovalbumin-binding peptides

| Name   | Sequence                        | Library origin | appK<sub>d</sub>** /μM |
|--------|---------------------------------|----------------|------------------------|
| OvaBP1 | MTTCFRSRKFWRYWCSWD              | NNK            | 1.548                  |
| OvaBP2 | MTTCRWRYGRFFRLLCSWD             | NNK            | 1.833                  |
| OvaBP3 | MTTCRSSDRPEGHRCWD               | VVN            | 0.085                  |
| OvaBP4 | MTTCASGRNKQANKCSWD              | VVN            | 0.065                  |
| OvaBP5*| MTTCKCRGICSRFSVGLG              | NNK            | -                      |
| OvaBP6 | MTTCTRRRSRWNWICSWD              | NNK            | 3.454                  |
| OvaBP7*| MTTCRLRMKVYRWRCWD               | NNK            | -                      |
| OvaBP8 | MTTCRRWWRLGLRNCSWD              | NNK            | 5.27                   |
| OvaBP9 | MNMWWQRSRRGKHLCSWD              | NNK            | 4.078                  |
| OvaBP10| MTTCYRKSFRMRYCSWD               | NNK            | 2.947                  |

* We met failure to synthesize these peptides.

** Because we observed multiphasic behavior for some OvaBPs, we averaged the obtained <i>K<sub>d</sub></i>s from each trace and reported them as apparent <i>K<sub>d</sub></i>s (app<i>K<sub>d</sub></i>s)
Figure Captions

Fig. 1 Translation time dependence of the FLAG sequence enrichment. (A) The illustration of the FLAG sequence used for the test selection. In the case of the JUNK sequence, the amino acid sequence DYKDDDDK was replaced to another longer sequence. (B) The FLAG sequence was effectively enriched by ribosome display using *E. coli* extract in all three translation time (5, 10 and 15 minutes) in sharp contrast to the control (1000-fold diluted input RNA was subjected to RT-PCR.). Each enriched population of FLAG sequence in the selections in 5, 10 and 15 minutes of translation was 82±2%, 86±2% and 87±11%, respectively.

Fig. 2 Library design for ribosome display using *E. coli* extract. A) In the codon table, the hydrophobic residues and the stop codons are shown in light gray and gray, respectively. B) The NNK codons cover 32 codons and are translated to the 20 canonical amino acids and TAG stop codon. C) The VVN codons cover 36 codons and are translated to 12 canonical amino acids (mostly hydrophilic or neutral amino acids). D) The illustration of the VVN and NNK libraries used for the selection of OVA binding peptides. E) The population of the sequence containing each number of VVN and NNK codons. The population of library before selection was calculated from occurrence probability of VVN and NNK codons. The read count and population of library after selection were obtained from NGS results at the end of eighth round of selection. Although the initial library contained over 90% VVN library, the library which was obtained after eight rounds of selection to OVA contained only 26% VVN origin sequences.

Fig. 3 (A) Dot blot assay clearly indicated that OvaBP6 specifically bind OVA and the
fluorescence signal of FAM-OvaBP6 clearly depend on the fixed amount of OVA. The dot blots of the other OvaBPs were shown in Fig. S5. B) The fluorescence intensity of each spot of OvaBP6, OvaBP8, OvaBP2 and OvaBP1 markedly increased in the respect of the fixed amount of OVA.

Fig. 4 The selected OvaBPs can bind to OVA even in the presence of serum. Solutions containing a series of concentration of serum, 50%, 25%, 10%, 5% and 0%, in the presence (upper row) and absence (lower row) of 5 mg/mL OVA were spotted. The two figures below each image represent the fluorescence intensity along the line profile for each image (black and grey profiles represent the line profiles in the presence and absence of OVA, respectively).
Figures

Fig. 1

A

| M | T | T | C | D | Y | K | D | D | D | D | K |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ATGACACACTGACTACAAGGATGACGATGACAAG |

T7p SD Helical linker SecM

B

| 5 min | 10 min | 15 min | Control |
|-------|--------|--------|---------|
| 15 20 25 30 35 | 15 20 25 30 35 | 15 20 25 30 35 | 15 20 25 30 35 |

PCR cycle

JUNK

FLAG
Fig. 2

A

B

C

D

E

| VN  | NNK | Population % | Read count | Population % |
|-----|-----|--------------|------------|--------------|
| 10  | 0   | 90.03        | 19515      | 26.23        |
| 0   | 1   | 0.25         | 2051       | 2.76         |
| 0   | 2   | 0.86         | 3032       | 4.30         |
| 0   | 3   | 1.79         | 9033       | 13.22        |
| 0   | 4   | 2.44         | 20474      | 27.52        |
| 0   | 5   | 2.27         | 13066      | 18.77        |
| 0   | 6   | 1.47         | 2002       | 5.19         |
| 0   | 7   | 0.66         | 663        | 0.99         |
| 0   | 8   | 0.19         | 65         | 0.09         |
| 0   | 9   | 0.03         | 12         | 0.02         |
| 0   | 10  | 0.003        | 2          | 0.003        |
| Others | | 100 | 74392 | 100 |

T7 p SD

Helical linker SaeM

X_{0.5}

ATGCAACATGT/VNV

ATGCAACATGT/NNK

Library before selection

Library after selection
Fig. 3

A

OvaBP6

OVA

BSA

10 5 2.5 1 0.5 0.25 0.01 μg

B

FL intensity

OVA conc./μg

2 4 6 8 10

OvaBP6

OvaBP8

OvaBP2

OvaBP1
Fig. 4
Graphical Index

E. coli extract

Short translation in ribosome display

Ovalbumin (Egg allergen)

Ovalbumin-binding peptides