



One-step immunoassay for food allergens based on screened mimotopes from autodisplayed F_V-antibody library

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ARTICLE INFO

Keywords:

One-step immunoassay

Mimotope

F_V-Antibody library

Autodisplay

Food allergen

ABSTRACT

One-step immunoassay detects a target analyte simply by mixing a sample with a reagent solution without any washing steps. Herein, we present a one-step immunoassay that uses a peptide mimicking a target analyte (mimotope). The key idea of this strategy is that the mimotopes are screened from an autodisplayed F_V-antibody library using monoclonal antibodies against target analytes. The monoclonal antibodies are bound to fluorescence-labeled mimotopes, which are quantitatively released into the solution when the target analytes are bound to the monoclonal antibodies. Thus, the target analyte is detected without any washing steps. For the mimotope screening, an F_V-antibody library was exhibited on the outer membrane of *E. coli* with a diversity of >10⁶ clones/library using autodisplay technology. The targeted clones were screened from the autodisplayed F_V-antibody library using magnetic beads with immobilized monoclonal antibodies against food allergens. The analysis of binding properties of a control strain with mutant F_V-antibodies composed of only CDR1 and CDR2 demonstrated that the CDR3 regions of the screened F_V-antibodies showed binding affinity to food allergens. The CDR3 regions were synthesized into peptides as mimotopes for the corresponding food allergens (mackerel, peanuts, and pig fat). One-step immunoassays for food allergens were demonstrated using mimotopes against mackerel, peanut, and pig fat without any washing steps in solution without immobilization of antibodies to a solid support.

1. Introduction

Immunoassays have been widely used for the detection of target analytes attributed to specific interactions between antibodies and antigens (target analytes) (Gan and Patel, 2013; Gosling, 2000). Immunoassays can be performed with different assay configurations, as shown in Fig. 1(a). The most widely used immunoassay configuration is based on sandwich complex formation. Here, antibodies are immobilized to a solid support, and the amount of target analytes is measured by the reaction of secondary antibodies labeled with reporting enzymes to produce a corresponding color change, fluorescence, or chemiluminescence signal that can be evaluated (Jung et al., 2021a; Kim et al., 2020, 2021; Zhao et al., 2009). In the configuration using non-labeled

detection, various types of transducers have been used to quantify the amount of target analytes bound to immobilized antibodies, such as surface plasmon resonance (SPR), quartz crystal microbalance (QCM), and electrochemical impedance spectrometry (EIS) (An et al., 2021; Bong et al., 2020; Farace et al., 2002; Jung et al., 2021b; Montagut et al., 2011). In both configuration types, washing steps are required before each reaction to remove unbound analytes or exchange reagents. A one-step immunoassay involves the detection of target analytes by mixing a sample with a reagent solution without any washing steps. The gold nanoparticles (AuNP) immobilized with detection antibodies have been reported to be used for one-step immunoassays. As the binding of specific antigens (target analytes) resulted in the aggregation of AuNPs, the size of aggregated complex was increased and the color was

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observed to be changed because the absorption wavelength was red-shifted. Such a shift of wavelength was correlated to the concentration of target analytes in samples (Liu et al., 2016; Yuan et al., 2011). Recently, one-step immunoassay based on switching peptides was reported. In this method, switching peptides were bound to the frame regions (FRs) of immunoglobulin G (IgG) which were known to have conserved sequences among IgGs. As the FRs were located within the antigen-binding pocket of IgGs, the switching peptides (labeled with fluorescence dyes) were quantitatively dissociated from the IgGs as the specific antigens (target analytes) to IgGs were bound to the antigen-binding pocket of IgGs. And the fluorescence signal was produced from the dissociated switching peptides quantitatively according to the amount of bound antigens. The one-step immunoassay based on switching peptides was demonstrated for human hepatitis surface antigen (hHBsAg) simply by mixing samples without any washing steps (Bong et al., 2021a).

In this study, we demonstrate a one-step immunoassay performed using a peptide mimicking a target analyte (mimotope). The mimotope was screened from an autodisplayed F_V-antibody library generated using monoclonal antibodies against the target analyte. In the one-step immunoassay configuration, the monoclonal antibodies were bound with fluorescence-labeled mimotopes, which were quantitatively released into the solution when the target analytes were bound to the monoclonal antibodies. Thus, the target analyte was detected without any washing steps. Here, the food allergen mimotopes were screened from an autodisplayed F_V-antibody library using monoclonal antibodies against the corresponding food allergens (mackerel, peanut, pig fat). The F_V-antibody represents the variable region (F_V) of the heavy chain (V_H) of immunoglobulin G (IgG), which is composed of three complementarity determining regions (CDRs) and frame regions (FRs). CDRs

directly interact with antigen epitopes, and these regions correspond to IgG paratopes (Herold et al., 2018; Polonelli et al., 2008; Zabetakis et al., 2013). A plasmid of the F_V-antibody library was produced by site-directed mutagenesis of the CDR3 region, which is composed of 11 amino acids, and the library was expressed on the outer membrane of *E. coli* using the autodisplay technology, as shown in Fig. 1(b) (Dao-Pin et al., 1987; Fortier et al., 2005; Gozalbo-Rovira et al., 2013). Autodisplay technology is an expression method that involves induction of the expression of a target protein (F_V-antibody library) on the outer membrane of *E. coli* as a fusion protein, with AIDA-1 used as an anchor protein. The genetic information of a target protein is inserted into the autodisplay vector as a passenger gene, which is expressed on the outer membrane of *E. coli* after transformation and IPTG induction (Jose and Meyer, 2007; Park et al., 2015). Using autodisplay technology, various kinds of proteins have been expressed on the outer membrane as target proteins, such as Z-domain, avidin, casein, lipase, Ro, and La antigens (Chang et al., 2017; Kim et al., 2016; Park et al., 2014b; Pyun et al., 2017; Yoo et al. 2011, 2017). Previous studies have shown that when the F_V-antibody library was autodisplayed on the outer membrane of *E. coli*, the target *E. coli* exhibiting binding activity toward antigens could be screened without repeated bio-panning. Because of the significantly high expression yield of $>10^5$ proteins/*E. coli* and a transformation yield of $>90\%$ for the total population, the target *E. coli* (a single *E. coli* cell) could be detected and isolated after the binding of fluorescence-labeled antigens to the F_V-antibodies on the outer membrane of *E. coli* cells (Dao-Pin et al., 1987; Fortier et al., 2005; Gozalbo-Rovira et al., 2013; McGuire et al., 2009; Wu et al., 2016). For the screening of mimotopes against food allergens, the F_V-antibody library was autodisplayed on the outer membrane of *E. coli* with a diversity of $>10^6$ clones/library. Next, the target clones with specific binding activity toward the monoclonal

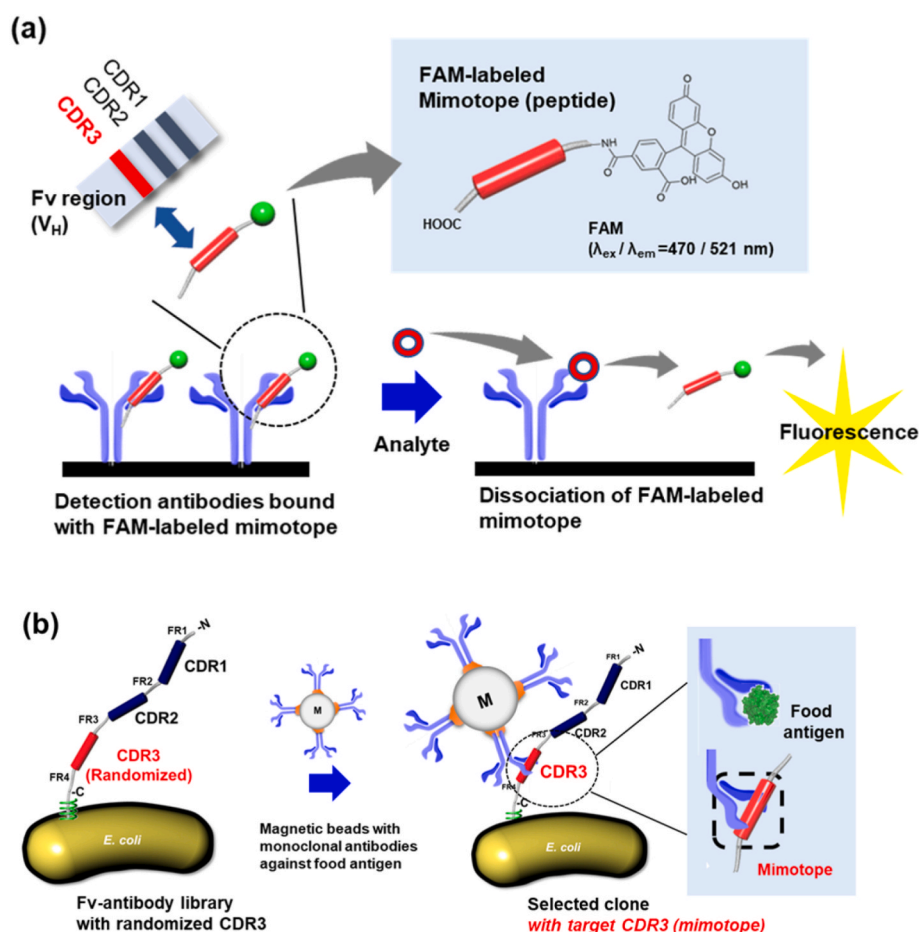


Fig. 1. One-step immunoassay. (a) Configurations of one-step immunoassay based on fluorescence-labeled mimotopes. (b) Screening of mimotopes from the F_V-antibody library. F_V-antibody library was prepared on the outer membrane of *E. coli* with randomized CDR3 region by site-directed mutagenesis, and target *E. coli* with F_V-antibody with binding affinity to the monoclonal antibody against food allergen was screened. The CDR3 region of screened F_V-antibody was recognized as a mimotope of the food allergen by the same monoclonal antibody.

antibodies against food allergens (mackerel, peanut, pig fat) were screened from the autodeisplayed F_V-antibody library. The target clone was separated from the F_V-antibody library using magnetic beads with immobilized monoclonal antibodies against food allergens. When the binding properties were analyzed using a control strain with mutant F_V-antibodies composed of only CDR1 and CDR2, the CDR3 regions of the screened F_V-antibodies were determined to have binding affinity to food allergens (Jung et al., 2021c; Lee et al., 2021). CDR3 regions with 11 amino acid residues were synthesized into peptides as mimotopes to the corresponding food allergens (mackerel, peanut, and pig fat).

A one-step immunoassay of food allergens was performed using these mimotopes as probes for the detection of food allergens in samples. A turn-on FRET reaction was used to generate the signal (Bong et al., 2021a). Fluorescence-labeled mimotopes were bound to monoclonal antibodies against food allergens. As the monoclonal antibodies were labeled with a fluorescence quencher, the fluorescence from the antibody-bound mimotopes could not be observed for the negative samples without food allergens. When food allergens in a sample were bound to the monoclonal antibodies, the fluorescence-labeled mimotopes dissociated into the solution, and fluorescence was observed. In this turn-on immunoassay, the detection of food allergens was made possible without any washing steps in the solution and immobilization of the antibodies to a solid support. Hence, the feasibility of one-step immunoassays for food allergens was demonstrated using three types of mimotopes corresponding to mackerel, peanuts, and pig fat.

2. Experimental

2.1. Materials

High-salt Luria-Bertani (LB) broth and LB agar were purchased from Duchefa Co. (Haarlem, Netherlands). Bovine serum albumin (BSA) and Tween® 20 were purchased from Sigma-Aldrich (Seoul, Korea). Dynabeads™ Protein G, BCA protein assay kit, high-fidelity Phusion polymerase, and PCR reagents were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). PCR Clean-up and NucleoSpin Gel kits were purchased from Macherey-Nagel Co. (Düren, Germany). Primers were synthesized by Macrogen Co. (Seoul, Korea). Klenow (exo-) polymerase was purchased from New England Biolabs (Ipswich, MA, USA). Food allergens from mackerel, peanuts, and pig fat, and monoclonal antibodies against food allergens were produced and supplied by the Gyeongsang National University (Jinju, Korea). Peptides were synthesized by Pepton Co. (Daejeon, Korea) with 90% purity. An Amicon® Ultra 0.5 mL filter with a cutoff of 10 kDa was purchased from Millipore Co. (Darmstadt, Germany). Phosphate buffered saline (PBS) (20X) was purchased from CUREBIO Co. (Seoul, Korea). The Tide Quencher™ 2 (TQ2) was purchased from AAT Bioquest (Sunnyvale, CA, USA).

2.2. Production of monoclonal antibodies against food allergens

Food allergens of mackerel, peanut, and pig fat were isolated via extraction and filtration, as previously reported (Kim et al., 2017). Six female BALB/c mice were immunized with each food allergen. For the first boosting, each food allergen at a concentration of 1.0 mg/ml (100 µL) was emulsified with an equal volume of Freund's complete adjuvant (100 µL), and the emulsified immunogen solution was injected into the peritoneal cavity of mice. The second and third boosting injections were administered at an interval of two weeks. Three days after the third injection, blood was collected from the tail vein of each mouse, and the titer of each antiserum was determined by indirect ELISA. The final boost injection was administered to mice exhibiting a high titer of anti-sera without the adjuvant (only 200 µL of immunogen). Three days after the final boost injection, spleen cells from immunized mice were collected and used for cell fusion with SP₂/0 myeloma cells. Cell fusion was performed as previously reported (Köhler and Milstein, 1975). Spleen cells (2.0×10^8 cells) were fused with SP₂/0 myeloma cells (2.0

$\times 10^7$ cells) using 50% PEG 1500 solution (1 mL). After HAT selection, the supernatant solution of the fused cells was analyzed by indirect ELISA to determine the antibody titer. The fused cells that were positively confirmed by indirect ELISA were selected and cloned using the limited dilution method. Monoclonal hybridoma cells producing monoclonal antibodies specific to the corresponding food allergen were screened using indirect ELISA. For the production of monoclonal antibodies, the female BALB/c mice were treated with pristine (0.5 ml), and the cloned hybridoma cells at a concentration of 1×10^7 cells were injected after 7–10 days. Ascites fluid was collected from the mice, and monoclonal antibodies were purified using saturated ammonium sulfate (Neoh et al., 1986). The purified monoclonal antibodies were then dialyzed in PBS (pH 7.4) for three days. The freeze-dried antibodies were stored at -20°C . The specificity and sensitivity of the monoclonal antibodies were confirmed by indirect ELISA and western blotting, respectively.

2.3. Preparation and screening of F_V-antibody library

The synthesized single-stranded primers containing the randomized oligonucleotide sequence of CDR3 (Table 1) and the corresponding reverse primers (Table 1) were mixed with NEB buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl₂, 100 µg/mL of BSA, pH 7.9) to obtain a total volume of 50 µL. After the annealing process (heating at 95°C for 5 min and cooling up to 36°C at a cooling rate of -0.3°C/s), Klenow (exo-) enzyme (3 µL), dNTPs (10 mM, 8 µL), and the NEB buffer were added to obtain a total volume of 200 µL for the extension reaction (37°C , 15 min). After inactivation of the extension reaction (75°C , 20 min), the PCR product (double-stranded F_V-antibody library primer with a randomized sequence of CDR3) was purified by a PCR Clean-up kit and a NucleoSpin® Gel. The method for autodeisplay was performed as previously reported (Jose and Meyer, 2007; Jung et al., 2021c; Lee et al., 2021). An F_V-antibody plasmid with a randomized sequence of the CDR3 region was prepared using PCR. The template plasmid pST009 (300 ng), double-stranded F_V-antibody library primer with a randomized sequence of CDR3 (2 µL), dNTPs (10 mM, 1 µL), HF buffer (10 µL), and Phusion high-fidelity polymerase (0.5 µL) were mixed to a total volume of 50 µL. PCR was performed using the following steps: (1) initial denaturation (98°C , 1 min), (2) denaturation (98°C , 30 s), (3) annealing (68°C , 1 min), (4) elongation (72°C , 5 min), (5) 30 cycles of (2)–(4), and (6) termination (72°C , 10 min). After PCR, the template plasmid was digested using the DpnI restriction enzyme (37°C , 16 h), and the digested PCR product was purified using a PCR Clean-up kit and NucleoSpin® Gel. Finally, the PCR product was filtered using an Amicon® Ultra 0.5 mL filter with a cutoff of 10 kDa. The autodeisplay of the F_V-antibody on the outer membrane of *E. coli* was performed by transforming the previously prepared autodeisplay vectors into competent *E. coli* BL21 (DE3) cells via electroporation. The transformed *E. coli* cells were cultured overnight by shaking in high-salt Luria-Bertani (LB) medium containing 50 mg/L kanamycin (37°C , 200 rpm, 16 h). Then, the culture was inoculated in LB medium mixed with EDTA (10 µM), kanamycin (50 mg/L), and β-mercaptoethanol (10 mM) at 37°C with shaking (200 rpm) until the culture demonstrated an OD_{600nm} of 0.5. The induction of F_V-antibody expression was initiated by adding 1 mM isopropylthio-β-galactoside (IPTG) at 30°C with mild shaking (120 rpm) for 3 h. After IPTG induction, centrifugation was performed for 3 min at $3000\times g$, and the sample was resuspended in an extraction buffer (2% Triton X-100, 50 mM Tris/HCl, 10 mM MgCl₂).

2.4. Screening of targeted *E. coli*

Screening of targeted *E. coli* using F_V-antibodies with a specific binding activity toward monoclonal antibodies against food antigens of mackerel, peanut, and pig fat was performed with the following steps: (1) Dynabeads™ Protein G (10 µL) and antibodies (1 mg/mL, 190 µL) against a single type of food allergen of mackerel, peanut, and pig fat

Table 1
Oligonucleotide primer sequences for FV-antibody Library.

Primer	Oligonucleotide sequence
Forward primer	5'-GTCTATTATTGCGCTCGT ¹ KRYVN ⁶ NVNNV ¹¹ NNVN ¹⁶ VNNVN ²¹ NVNNV ²⁶ NNGAT ³¹ KW ³³ Y TGGGGTCAAGGTACTACGGTTACG-3'
Reverse primer	3'-CCCAGTTCATGATGCCAATGC-5'
Nucleotide compositions	N = A, C, G, T / R = A, G / K = G, T / Y = C, T / W = A, T / V = A, C, G

were mixed with 0.1% PBST (0.1% Tween 20) for 1 h (2) The monoclonal antibody-bound magnetic beads were sorted using a magnet, and unbound monoclonal antibodies were washed five times with 0.1% PBST and PBS. (3) The monoclonal antibody-bound magnetic beads were then mixed with the F_V-antibody library (100 μ L, OD₆₀₀ = 1.0) at 4 °C and 15 rpm for 1 h (4) The monoclonal antibody-bound magnetic beads with bound *E. coli* were separated using the magnet and washed with 0.1% PBST and PBS ten times. (5) Separated monoclonal antibody-bound magnetic beads with bound *E. coli* were finally resuspended in LB medium (50 μ L), and then the separated magnetic beads were spread on agar plates to obtain *E. coli* clones. The binding properties of *E. coli* bound with the F_V-antibody toward antibodies against food allergens of mackerel, peanut, and pig fat were evaluated via immunostaining. The screened *E. coli* were mixed with FITC-labeled antibodies against food allergens of mackerel, peanut, and pig fat for 1 h with mild shaking. After centrifugation (3000 $\times$ g, 3 min), the *E. coli* was washed with PBS thrice, and the fluorescence images were captured using a fluorescence microscope (Model: BX51) purchased from Olympus Co. (Tokyo, Japan). The quantitative binding of *E. coli* with the F_V-antibody to antibodies against food allergens of mackerel, peanut, and pig fat was performed using a FACSCalibur flow cytometer (Becton–Dickinson, Franklin Lakes, NJ, USA). The antibodies against food allergens were labeled with fluorescein, and the antibody solution was mixed with each screened clone (OD_{600 nm} = 1.0, 100 μ L) with concentrations ranging from 0.3 to 10 μ g/mL for 1 h with mild shaking. Then, *E. coli* were washed thrice to remove the unbound antibodies by centrifugation (3000 $\times$ g, 3 min), and the pellet was resuspended in PBS buffer. Flow cytometry measurements were performed at an excitation wavelength (λ_{EX}) of 488 nm and with the threshold setting of side scatter to remove the background noise signal to analyze the signal from *E. coli*.

2.5. One-step immunoassay

Antibodies were labeled with a quencher by inducing a reaction of the antibody solution (1 mg/ml, 200 μ L) and Tide Quencher™ 2 (TQ2) (10 mg/ml, 30 μ L) for 4 h with mild shaking. The unreacted quencher was removed using a desalting column. The binding of fluorescent-labeled mimotope was performed by mixing a quencher-labeled antibody solution mixture (250 μ g/ml, 250 μ L) and fluorescence-labeled mimotope solution (150 μ M, 25 μ L) for 2 h with mild shaking. The unreacted quencher was removed using a desalting column. A one-step immunoassay was performed by mixing the mimotope complex solution (20 μ g/ml, 30 μ L) and the sample solution (160 μ L) for 1 h. The fluorescence signal was measured at an excitation wavelength of 470 nm and an emission wavelength of 521 nm using a Denovix QFX Fluorometer (Denovix Inc., Wilmington, DE, USA). The limits of detection (LOD) was determined as the average intensity plus three standard deviations (3 σ) from the blank sample (deionized water, DW) and the dynamic range was calculated by linearly correlating with the logarithmic concentration of TSSPs from the LOD (Ezan et al., 2000). The commercial lateral flow immunoassay kits for the detection of peanut and pig fat were purchased from Neogen (Lansing, MI, USA).

3. Results and discussion

3.1. Screening of the F_V-antibody library

The F_V-antibody library was prepared from the F_V region of a heavy chain (V_H) of immunoglobulin (IgG), which was composed of three CDR regions and four FR regions. The F_V-antibody library was constructed via site-directed mutagenesis of the CDR3 region with 11 amino acids. The CDR3 has been conventionally used for the F_V-library construction in the phage display technology because CDR3 region has longer amino acid sequence of 11 residues for the randomization in comparison with CDR1 (7 residues) and CDR2 (5–6 residues) [(Ferrari et al., 2020)]. Additionally, the randomized CDR3 was efficiently used for the screening of targeted clones with a specific affinity to antibodies using a mutant clone with the constant amino acid sequences of only CDR1 and CDR2 (without CDR3). As shown in Fig. 2(a), the synthesized forward primers (75 bp in total) together with a randomized CDR3 region (33 bp) were annealed with the reverse primers (22 bp) (Dao-Pin et al., 1987; Fortier et al., 2005; Gozalbo-Rovira et al., 2013). As shown in Fig. 2(b), the plasmid of the F_V-antibody library was prepared using a template plasmid (pST009, 5169 bp). The template plasmid was digested using *DpnI*. The prepared plasmid comprised genetic information required for the autodisplay of the F_V-antibody library, consisting of three CDR regions, four FR regions, such as T7-promotor, signal peptide, and beta-barrel sequence (Jose and Meyer, 2007). Primer sequences are summarized in Table 1. As shown in Fig. 2(c), F_V-antibodies with randomized CDR3 regions were autodisplayed on the outer membrane of *E. coli* as fusion proteins comprising the AIDA-1 protein (beta-barrel). A negative control strain with autodisplayed mutant F_V-antibodies with only CDR1 and CDR2 regions was also prepared (Jung et al., 2021c; Lee et al., 2021). The diversity of the autodisplayed F_V-antibody library was estimated to be 2.8×10^5 clones/library from a random selection of cultured clones. As shown in Fig. 2(d), the expression of F_V-antibodies on the outer membrane of *E. coli* (64.2 kDa) was confirmed via SDS-PAGE, and the yield of expression was estimated to be 2.02×10^5 proteins/*E. coli* via densitometry.

Food allergens were isolated as thermostable soluble proteins (TSSPs) corresponding to mackerel, peanuts, and pig fat from the boiled supernatant (Jeong-Sook et al., 2016; Park et al., 2014a), and monoclonal antibodies were produced using these food allergens (Jeong-Eun et al., 2017; Jeong-Sook et al., 2016; Kim et al., 2017; Park et al., 2014a). For the screening of F_V-antibodies with the binding affinity toward monoclonal antibodies against food allergens, magnetic beads with immobilized monoclonal antibodies against food allergens were mixed with the *E. coli* with autodisplayed F_V-antibody library, as shown in Supplementary Fig. S1. After isolation of the magnetic beads with bound *E. coli* using an external magnet, the bacteria were cultured on agar plates. The screened *E. coli* were analyzed using fluorescence-labeled monoclonal antibodies against the corresponding food allergen. The fluorescence signal from *E. coli* represented the specific binding of fluorescence-labeled monoclonal antibodies to the autodisplayed F_V-antibodies on the outer membrane of the screened *E. coli*. Finally, the genetic sequences of the CDR3 region of the screened *E. coli* was analyzed and compared with the template sequence of the CDR3 region, which was used for site-directed mutagenesis.

The genetic information of the CDR3 region was analyzed for the F_V-

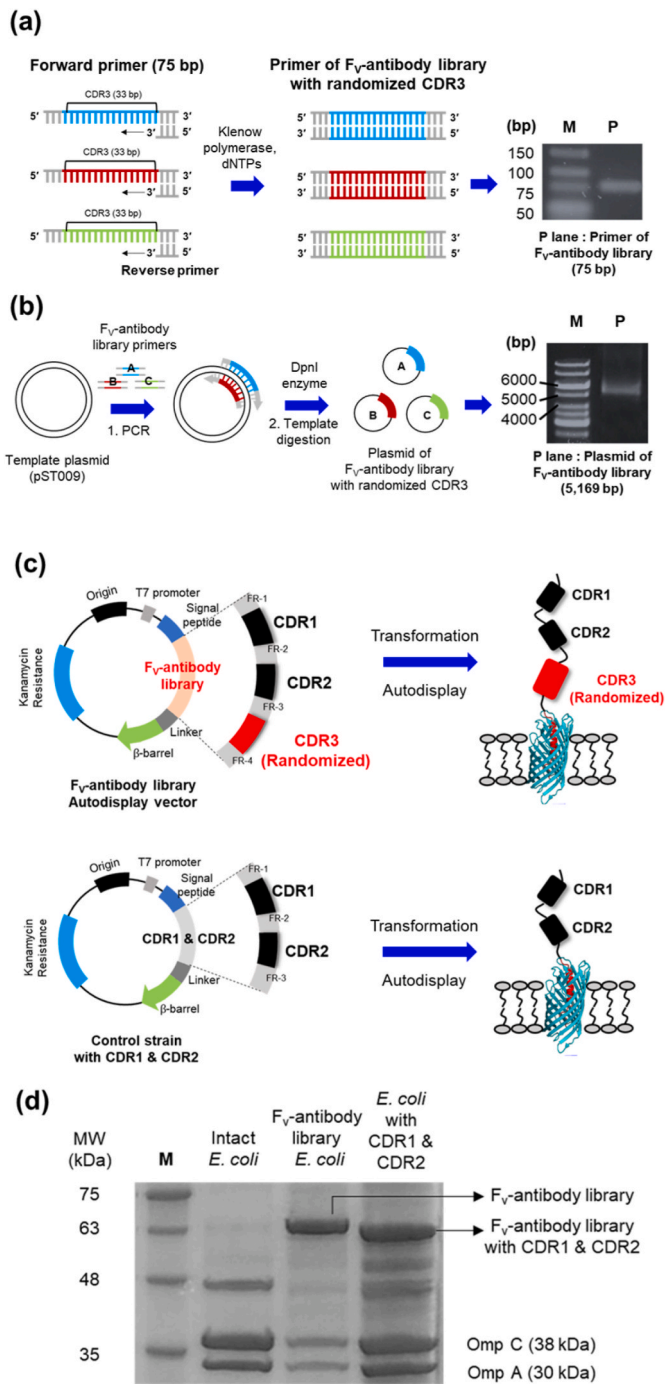


Fig. 2. Preparation of the F_V-antibody library. (a) Site-directed mutagenesis of CDR3 region, (b) Preparation of the plasmid for autodisplay of the F_V-antibody library, (c) Autodisplayed F_V-antibody and a control strain with only two CDR regions (CDR1 and CDR2), (d) Comparison of the autodisplayed proteins on the outer membrane of *E. coli*.

antibody with a binding affinity toward the monoclonal antibody against the TSSP of mackerel, as shown in Fig. 3(a). Magnetic beads with antibodies against the TSSP of mackerel were used to screen *E. coli* with the F_V-antibody library, which were then cultured on an agar plate. Several clones were derived from the agar plate and cultured again, and fluorescence-labeled monoclonal antibodies against the TSSP of mackerel were reacted with the cultured *E. coli*. Flow cytometry showed that the binding of fluorescence-labeled antibodies was observed in five clones in comparison with intact *E. coli* without autodisplayed F_V-

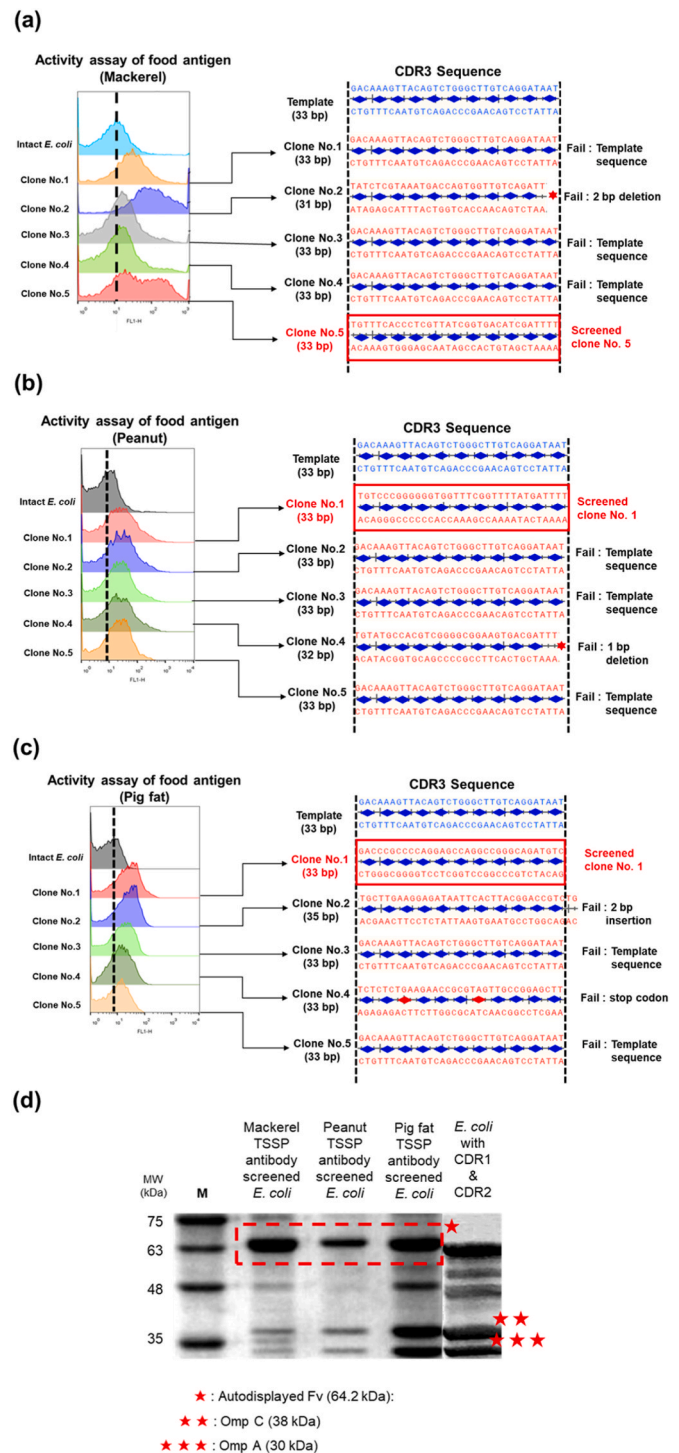


Fig. 3. Genetic sequence of CDR3 regions of screened *E. coli* with a binding affinity toward monoclonal antibodies against food antigens isolated from (a) mackerel, (b) peanut, (c) pig fat. (d) SDS-PAGE of proteins on the outer membrane of selected clones and the control strain only with autodisplayed CDR1 and CDR2.

antibodies on the outer membrane. After sequencing of the screened five clones, the nucleotide sequences of CDR3 regions were compared with the template plasmid (pST009), and only clone no. 5 exhibited the adequate desired sequence. In the case of clone nos. 1, 3, and 4, the nucleotide sequences were estimated to be identical to the template sequence. In the case of clone no. 2, two base pairs were deleted from the CDR3 region. Based on these results, only clone no. 5 showed the

autodisplayed F_V-antibody with a binding affinity toward the antibodies against the TSSP of mackerel.

The genetic information of the CDR3 region was analyzed for the F_V-antibody with binding affinity to the monoclonal antibody against the TSSP of peanut, as shown in Fig. 3(b). The target *E. coli* was screened using magnetic beads with monoclonal antibodies against the TSSP of peanuts. Flow cytometry showed that the binding of fluorescence-labeled antibodies was observed in five clones in comparison with intact *E. coli*. After sequencing of the five screened clones, the nucleotide sequences of CDR3 regions were compared with the template plasmid (pST009), and only clone no. 1 exhibited the desired sequence. In clone no. 2, 3, and 5, the nucleotide sequences were estimated to be identical to the template sequence. In the case of clone no. 4, one base pair was deleted from the CDR3 region. These results showed that clone no. 1 autodisplayed the F_V-antibody with a binding affinity toward the antibodies against the TSSP of peanuts.

The genetic information of the CDR3 region was analyzed for the F_V-antibody with a binding affinity toward the monoclonal antibody against the TSSP of pig fat, as shown in Fig. 3(c). The target *E. coli* was screened using magnetic beads with monoclonal antibodies against other food allergens. Flow cytometry showed that the binding of fluorescence-labeled antibodies was observed in five clones in comparison with intact *E. coli*. After sequencing of the five screened clones, the nucleotide sequences of the CDR3 regions were compared with the template plasmid (pST009), and only clone no.1 exhibited the adequate desired sequence. In the case of clone no. 2, two base pairs were inserted in the CDR3 region. In the case of clones 3 and 5, the nucleotide sequences were estimated to be identical to the template sequence. In the case of clone no. 4, the nucleotide sequence included a stop codon. These results showed that clone no. 1 autodisplayed the F_V-antibody with a binding affinity toward the antibodies against the TSSP of pig fat. As shown in Fig. 3(d), the expression of F_V-antibodies on the outer membrane of *E. coli* (64.2 kDa) was confirmed by SDS-PAGE, and the yields of expression were 3.41×10^5 , 2.54×10^5 , and 1.59×10^5 proteins/*E. coli* for the TSSPs of mackerel, peanuts, and pig fat, respectively, as estimated via densitometry.

3.2. Synthesis of mimotopes from screened F_V-antibodies

The screened *E. coli* exhibited autodisplayed F_V-antibodies with a binding affinity toward the monoclonal antibodies against food antigens. As the autodisplayed F_V-antibodies had three CDR regions and four FR regions, the specific region required for the binding of food allergens needed to be determined within the F_V-antibodies. These regions of the F_V-antibodies could bind to the monoclonal antibodies as in the case of the food allergens, which are known as “mimotopes” (peptides mimicking specific food allergens). To evaluate the specific region of F_V-antibody for binding to monoclonal antibodies (mimotope candidates), the binding properties of the screened clones were analyzed in comparison with native *E. coli* and a control strain with mutant F_V-antibodies composed of only CDR1 and CDR2 regions.

The binding affinity of the screened *E. coli* toward the monoclonal antibodies against food antigens was compared to that of the native *E. coli* and a control strain with only CDR1 and CDR2 regions using immunostaining. As shown in Fig. 4(a), the screened *E. coli* clone (clone no. 5) was made to react with fluorescence-labeled monoclonal antibodies against the TSSP of mackerel. The intact *E. coli* without autodisplayed F_V-antibodies, and *E. coli* with only two CDR regions (CDR1 and CDR2), were also caused to react with the antibodies under the same conditions. The screened *E. coli* showed a strong fluorescence signal in comparison with the native *E. coli* and the control strain. As shown in Fig. 4(b) and (c), the screened *E. coli* clones with a binding affinity toward monoclonal antibodies against the TSSP of peanut (Clone no. 1) and pig fat (Clone no. 1) showed strong fluorescence when fluorescence-labeled monoclonal antibodies against the TSSPs of peanut and pig fat were reacted. In the case of native *E. coli* and the control strain, no significant fluorescence signal was observed. These results indicated that the F_V-antibodies on the screened *E. coli* clones showed a specific binding affinity toward monoclonal antibodies against TSSPs of food allergens of mackerel, peanuts, and pig fat via interaction with the CDR3 region of the F_V-antibodies of the screened clones.

The proportion of *E. coli* with a binding affinity toward monoclonal antibodies against food antigens was quantitatively analyzed in the whole bacterial population using flow cytometry. As shown in Fig. 5(a),

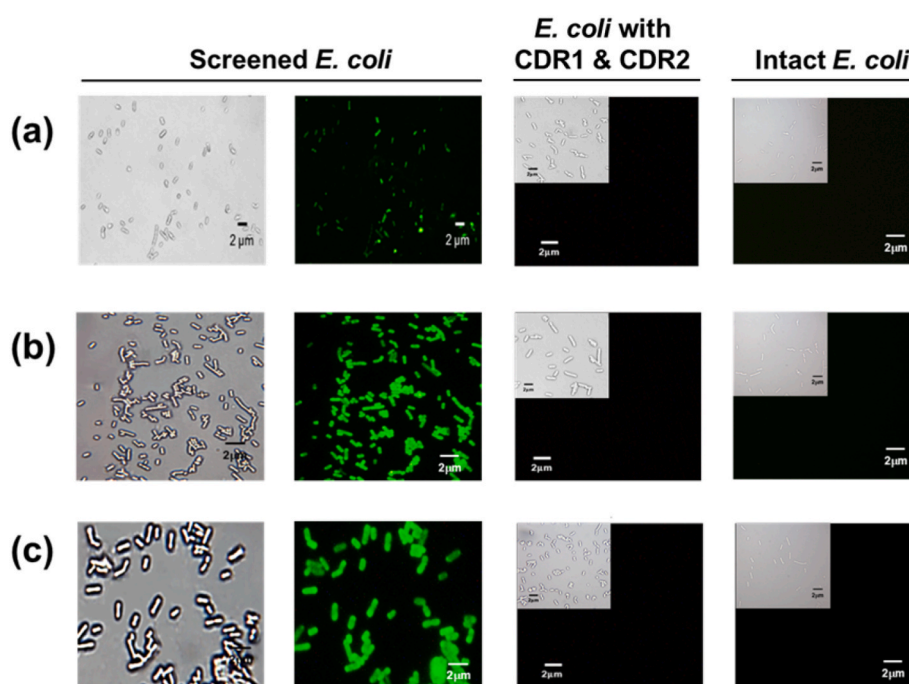


Fig. 4. Fluorescence immunostaining of screened *E. coli* with monoclonal antibodies against food antigens isolated from (a) mackerel, (b) peanut, (c) pig fat. The images were compared with native *E. coli* and a control strain with only two CDR regions (CDR1 and CDR2).

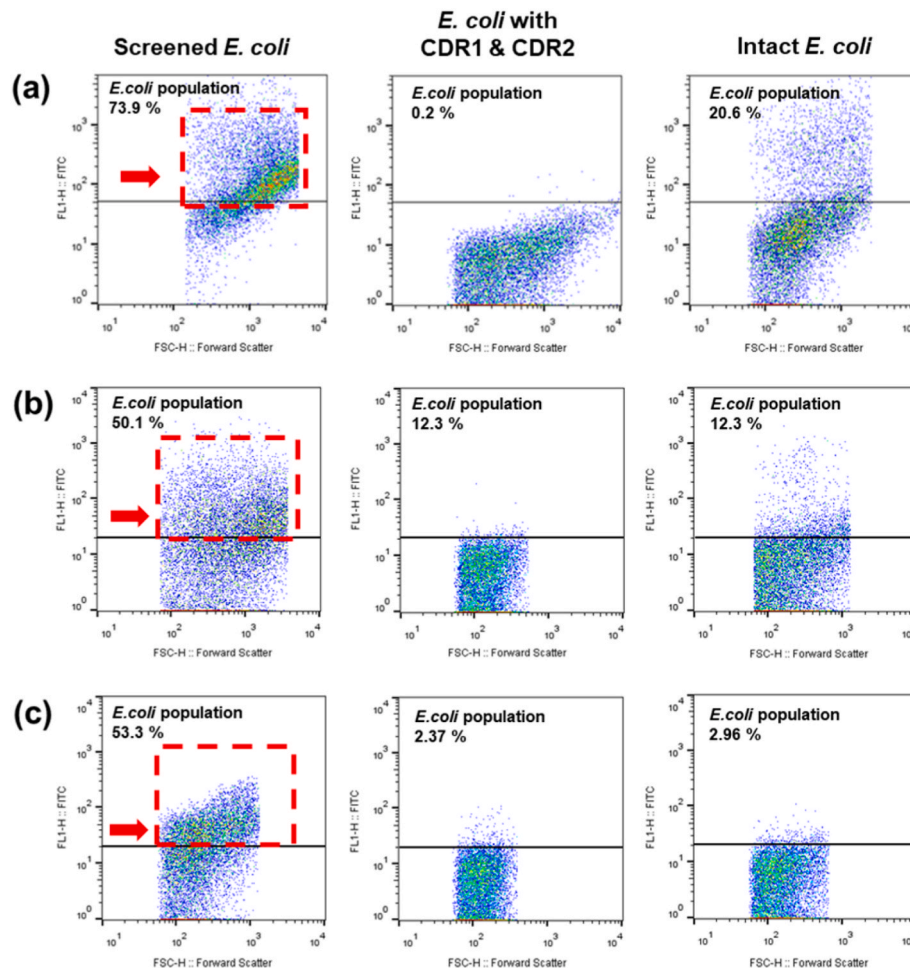


Fig. 5. Flow cytometric analysis of the binding affinity of Fluorescence binding test of the screened *E. coli* with monoclonal antibodies against food antigens from (a) mackerel, (b) peanut, (c) pig fat. The results of flow cytometry for screened *E. coli* were compared to those of native *E. coli* and a control strain with only two CDR regions (CDR1 and CDR2).

the screened *E. coli* clone no. 5 showed a strong fluorescence signal when reacted with the fluorescence-labeled monoclonal antibodies against the TSSPs of mackerel. *E. coli* at a proportion of 73.9% of the whole population demonstrated a high fluorescence signal of more than 50 (AU). Under the same reaction conditions as the fluorescence-labeled monoclonal antibodies, a relatively lower proportion of the native *E. coli* without autodisplayed F_V-antibodies (0.2%) and the control with only two CDR regions (CDR1 and CDR2) (20.6%) showed a fluorescence signal of more than 50 (AU). These results showed that F_V-antibodies on the screened *E. coli* clone (clone no. 5) had a specific binding affinity toward monoclonal antibodies against the TSSP of mackerel. The binding was observed between the CDR3 region of F_V-antibodies of the screened clone (clone no. 5) and the monoclonal antibodies against the TSSP of mackerel. As shown in Fig. 5(b) and (c), the screened *E. coli* clones with a binding affinity toward monoclonal antibodies against the TSSP of peanut (Clone no. 1) and pig fat (Clone no. 1) were treated with fluorescence-labeled monoclonal antibodies against the TSSPs of peanut and pig fat. The proportion of *E. coli* with higher cutoff fluorescence signals was estimated to be 50.1% for TSSPs of peanut and 53.3% for TSSPs of pig fat among the total *E. coli* population. However, the native *E. coli* and the control strain showed that a far lower proportion of *E. coli* showed a fluorescence signal higher than the cutoff value. These results also showed that F_V-antibodies on the screened *E. coli* clones had a specific binding affinity for the corresponding monoclonal antibodies, and the binding was observed between the CDR3 region of F_V-antibodies of the screened clone and the monoclonal antibodies.

The binding affinity of the screened *E. coli* toward the monoclonal antibodies against food antigens was quantitatively analyzed using flow cytometry. As shown in Fig. 6(a), the screened clone was treated with fluorescence-labeled anti-TSSP (mackerel) antibodies in the concentration range of 0.3–10 µg/ml. Three types of negative controls, intact *E. coli* without the autodisplayed F_V-antibodies, *E. coli* with only two CDR regions (CDR1 and CDR2) and *E. coli* with autodisplayed F_V-antibodies of template sequence (template F_V) were used for flow cytometry. In the case of the screened *E. coli* clone, the fluorescence signal from *E. coli* was observed to increase with the concentration of the treated antibodies. In the case of the three kinds of negative controls, no significant fluorescence signal was observed even after treatment with fluorescence-labeled antibodies. Based on the standard curve of flow cytometry, the binding constant (K_D) of screened F_V-antibodies on the outer membrane of *E. coli* was estimated to be 2.7 ± 1.2 (n=3) µg/ml. As shown in Fig. 6(b) and (c), the screened clones for peanut and pig fat antigens were treated with fluorescence-labeled anti-TSSP antibodies of peanut and pig fat in the concentration range of 0.3–10 µg/ml. Three types of negative controls, native *E. coli*, a control strain and *E. coli* with autodisplayed F_V-antibodies of template sequence (template F_V), were also used for flow cytometry. In the case of the screened *E. coli* clones, the fluorescence signal from *E. coli* was observed to increase with the concentration of the treated antibodies. In the case of two kinds of negative controls, there was no significant fluorescence signal even after treatment with fluorescence-labeled antibodies. From the standard curve of flow cytometry, the binding constant (K_D) of screened F_V-

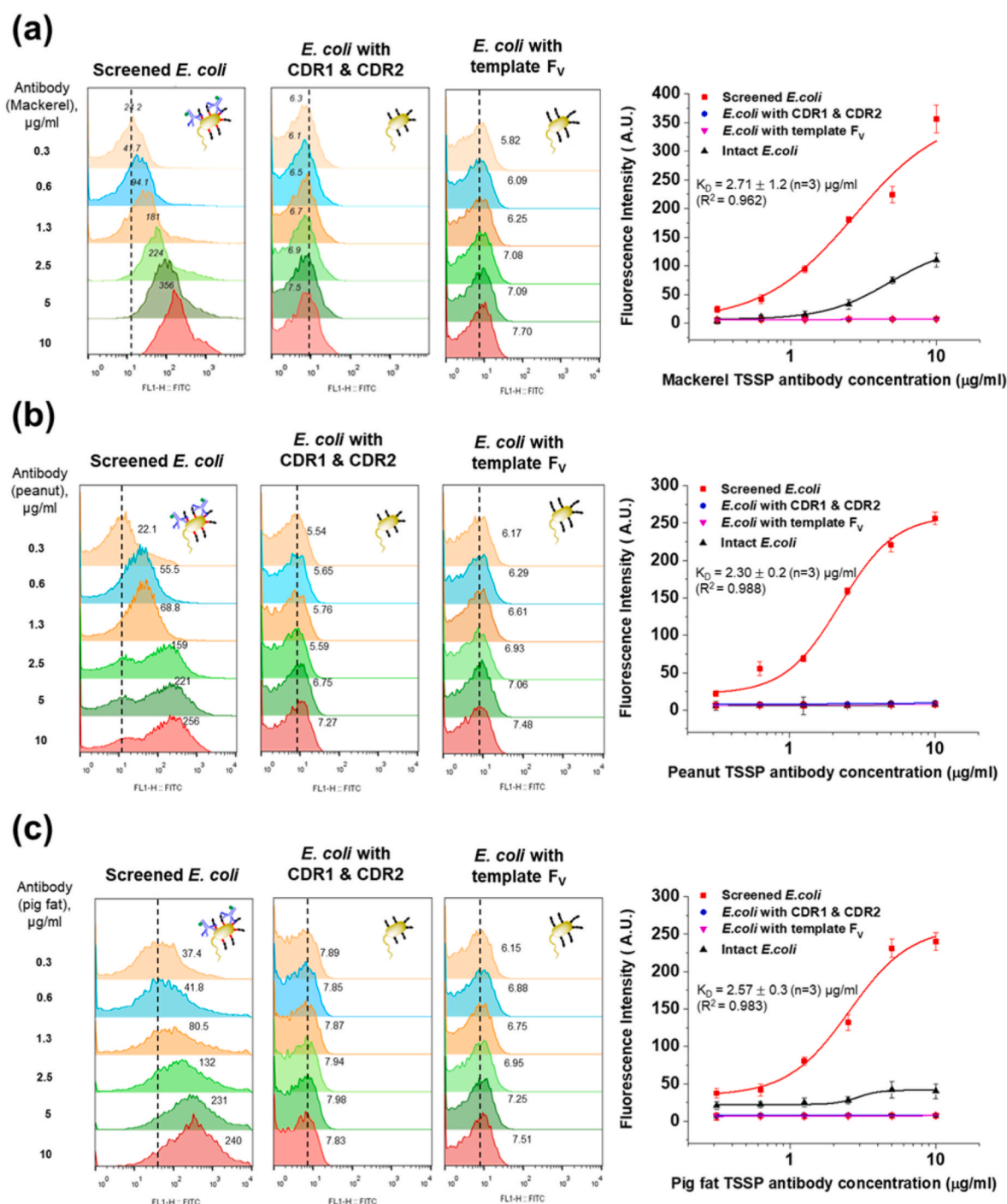


Fig. 6. Analysis of quantitative binding affinity of autodisplayed F_V -antibodies to monoclonal antibodies against food antigens isolated from (a) mackerel, (b) peanut, (c) pig fat. The binding constant (K_D) was estimated by fitting with the isotherm model.

antibodies on the outer membrane of *E. coli* was estimated to be 2.3 ± 0.2 ($n = 3$) $\mu\text{g/ml}$ for the TSSP of peanuts and 2.6 ± 0.3 ($n = 3$) $\mu\text{g/ml}$ for the TSSP of pig fat. These results showed that (1) the screened F_V -antibodies could quantitatively bind to the corresponding monoclonal antibodies against food allergens, and (2) the CDR3 regions of the screened F_V -antibodies determined the binding specificity toward monoclonal antibodies against food allergens.

The comparison with native *E. coli*, *E. coli* with autodisplayed F_V -antibodies of template sequence (template F_V) and a negative control strain with F_V -antibodies composed of CDR1 and CDR2 regions showed that the CDR3 region of the screened clones directly interacted with the

monoclonal antibodies against TSSPs of food allergens. Based on these results, the CDR3 regions were determined to be mimotopes (mimicking peptides) of food allergens, and the CDR3 regions of the screened clones (11 amino acids) were synthesized into peptides with 15 amino acid residues by inserting two amino acid residues at both sides of CDR3 regions (11 residues). The C-terminal was labeled with a fluorescein molecule, as summarized in Table 2.

3.3. One-step immunoassay using mimotopes of food allergens

The synthesized mimotopes were used for the one-step immunoassay

Table 2

Oligonucleotide primer and peptide sequences for screened mimotopes from FV-antibody Library.

Food antigen		Sequences	KD (μ M)
Mackerel	Oligonucleotide	5' - 1TGTTT6CACCC11TCGTT16ATCGG 21TGACA26TCGAT31TT33T -3'	-
	Mimotope (peptide)	1CFTLV 6IGDID 11F	3.60 $\pm$ 0.8
Peanut	Oligonucleotide	5' - 1TGTC6CGGG11GGTGG16TTTCG 21GTTTT26ATGAT31TT33T -3'	-
	Mimotope (peptide)	1CPGGW 6FRFYD 11F	8.42 $\pm$ 0.6
Pig fat	Oligonucleotide	5' - 1GACCC6GCC11AGGAG 16CCAGG21CCGGG26CAGAT31GT 33C- 3'	-
	Mimotope (peptide)	1DPPQE 6PGRAD 11V	5.94 $\pm$ 0.6

of food allergens, which involved the detection of food allergens requiring only sample treatment without any washing steps. Food allergens are naturally occurring proteins in foods or their derivatives that induce abnormal immune responses called allergic reactions (Broide, 2001; Lee et al., 1995; Okunuki et al., 2000). The level of allergic reactions to foods varies widely, ranging from mild discomfort to asthma and anaphylaxis. Many different methods have been used to identify food allergens (Nakamura et al., 2005). Usually, food allergens are detected using immunoassays that are based on antibodies. Conventional immunoassays, such as ELISA, require washing steps between sample treatments and reagents (Gosling, 2000). Even if non-labeled detection is applied, the washing step is required to rinse out the unbound samples, as shown in Fig. 1(a) (Bong et al., 2021b; Lee et al., 2021). As previously mentioned at introduction, the one-step immunoassay could be made using switching peptides which dissociated after binding of target analytes of IgGs according to the concentration of target analytes. As the switching peptides were bound to the FRs of IgGs, these switching peptides could be used for any IgGs regardless of the antigenic specificity of each IgG. However, the mimotope was screened to have a specific binding affinity to a specific monoclonal antibody (IgG) and it could bind only to the specific IgG. As demonstrated in this work, the one-step immunoassay was feasible using screened mimotopes from Fv-library. Additionally, the mimotope could be used as a positive control antigen for the specific monoclonal antibody (IgG). For example, the mimotope for the monoclonal antibody (IgG) against mackerel could be used as a positive control antigen, although the monoclonal antibodies were made from the mixture of antigens (TSSPs of mackerel).

The homology of the amino acid sequence of CDR3 regions (mimotopes) from the screened clones was analyzed using BLAST software (<https://blast.ncbi.nlm.nih.gov>). In the case of the mackerel mimotope, amino acid sequences similar to the CDR3 region of screened clone were found in a few types of proteins in microorganisms, such as *Shewanella* spp., *Vibrio* spp., and *Desulfobacterium* spp., as summarized in Table 3. These bacterial strains are known to persist in many marine and estuarine environments (Grimes et al., 2009; Wang et al., 2020). In particular, the CDR3 region of mimotopes of mackerel was highly similar to the GGDEF family protein called diguanylate cyclase, which has been reported in these bacterial strains (Grimes et al., 2009). Diguanylate cyclase is an enzyme that produces the secondary messenger c-di-GMP, which is associated with biofilm formation and contributes to the spoilage activity of these bacterial strains (Wang et al., 2020). In the case of the peanut mimotope, amino acid sequences similar to the CDR3 region of the screened clone were found in a few types of proteins in a parasite species called *Trichinella* spp. These parasite strains cause a parasitic disease called trichinellosis, which affects human intestines and results in diarrhea, abdominal pain, and vomiting. Trichinellosis is also a significant concern for porcine animal production and food safety

Table 3

Homology analysis of amino acid sequences of food allergens.

Food allergen	Amino acid sequence	Sequence ID *
Mackerel	CFTLVIGDIDF	
GGDEF domain-containing protein from <i>Vibrio lentus</i>	308-TLVIGDIDF- 316	WP_102541631.1
GGDEF domain-containing protein from <i>Desulfobacterium autotrophicum</i>	316-TLVIGDIDF- 324	WP_041274252.1
Sensor domain-containing Diguanylate cyclase from <i>Shewanella algae</i>	317-FSLVIGDIDF- 326	TWO84978.1
Peanut	CPGGWFRFYDF	
Hypothetical protein D6791_06340 from <i>Chloroflexi</i>	117-GGWFRFYD- 124	HEX42681.1
Hypothetical protein T01_10819 from <i>T. spiralis</i>	278-WFRFYDF- 284	KRY38992.1
Hypothetical protein T03_5340 from <i>T.</i> <i>Britovi</i>	278-WFRFYDF- 284	KRY50023.1
Pig fat	DPPQEPRADF	
SAM-dependent methyltransferase protein from <i>Rhodopseudomonas</i> <i>palustris</i>	264- DPPQEPRADF- 274	WP_184262890.1
SAM-dependent methyltransferase protein from <i>Rhodopseudomonas</i> <i>Rhenobacensis</i>	264- DPPQEPRADF- 274	WP_011471879.1

* Source: GenBank(www.ncbi.nlm.nih.gov/genbank/)

(Gottstein et al., 2009). This parasitic infection in the intestine, called enteric helminth infection, exhibits stimulatory as well as suppressive effects on allergic responses (McSorley et al., 2019). Additionally, the peanut mimotope was found in a few types of proteins in *Chloroflexi*. This phylum of bacteria is one of the dominant phyla in the peanut rhizosphere, which involves the soil volume surrounding the immediate surface of the peanut root (Dai et al., 2019; Hütsch et al., 2002). In the case of the mimotope of pig fat, amino acid sequences similar to the CDR3 region of the screened clone were found in a few kinds of proteins in a purple, rod-shaped, gram-negative, non-sulfur bacterium called *Rhodopseudomonas* spp. These bacterial strains are widely distributed in nature and have been isolated from diverse sources such as swine waste lagoons, earthworm droppings, marine coastal sediments, and pond water (Larimer et al., 2004). The BLAST search showed that the CDR3 region of mimotopes of pig fat was highly similar to the SAM-dependent methyltransferase protein which was reported to be found in these bacterial strains. Such Bacterial infection in the intestine, called enteric helminth infection, has been shown to have stimulatory as well as suppressive effects on allergic responses (Larimer et al., 2004). These results indicated that the amino acid sequence of mimotopes contained information about proteins closely related to food allergens rather than a random selection from the Fv-antibody library. Therefore, the screening of mimotopes against monoclonal antibodies from the Fv-antibody library could be used as an effective analytical tool for the investigation of food allergens.

One-step immunoassays for the quantitative analysis of TSSPs were performed using the mimotopes of TSSPs. For the one-step immunoassay, anti-TSSP antibodies were immobilized on the microplate and the corresponding mimotopes labeled with fluorescein were bound to the paratopes of immobilized monoclonal antibodies, as shown in Fig. 7(a). When a positive sample with the corresponding antigens was reacted, the pre-bound mimotopes (with fluorescence labels) were released according to the concentration of antigens in the sample. In the case of negative samples without the corresponding antigens, there was no change in fluorescence. In order to show the binding of fluorescence labeled mimotopes to the immobilized detection antibodies, the fluorescence signal was measured when mimotope labeled with FAM ($\lambda_{\text{ex}} = 492 \text{ nm}$, $\lambda_{\text{em}} = 521 \text{ nm}$) were treated to the immobilized antibodies. As shown in Fig. 7(b), the fluorescence signal was observed to be significantly increased which represented the binding of mimotopes to the immobilized detection antibodies. Using this assay configuration, the

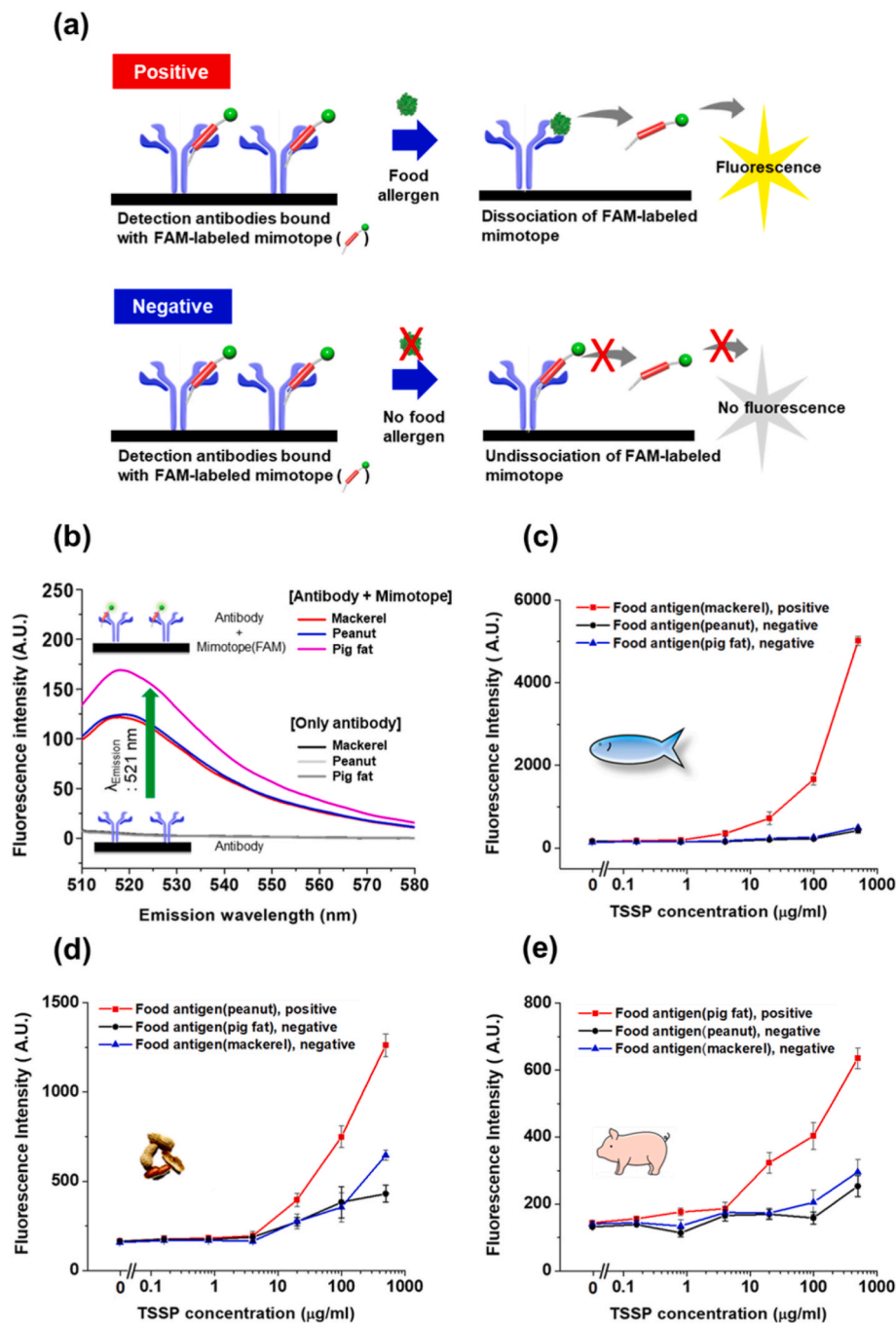


Fig. 7. One-step immunoassay based on mimotopes with immobilized monoclonal antibodies. (a) Configuration of one-step immunoassay. (b) Fluorescence spectrum of negative samples with FAM-labeled mimotopes and without FAM-labeled mimotopes ($\lambda_{\text{ex}} = 492 \text{ nm}$, $\lambda_{\text{em}} = 521 \text{ nm}$). Standard curves for the one-step immunoassay of food antigens isolated from (c) mackerel, (d) peanut, (e) pig fat.

concentration of food allergens could be estimated by mixing the samples without any washing process. As shown in Fig. 7(c)–(e), a one-step immunoassay of TSSPs of mackerel, peanut, and pig fat was demonstrated using the immobilized antibodies against TSSPs with mimotopes of corresponding TSSPs. In the case of the mackerel, a one-step immunoassay was possible in the TSSP concentration range of 0.16–500 μg/ml. For TSSPs of peanut and pig fat, one-step immunoassay was possible in the TSSP concentration range of 0.16–500 μg/ml and 0.16–500 μg/ml, respectively. These results showed that one-step immunoassays for the quantitative analysis of TSSPs were possible using the mimotopes of TSSPs.

Another type of one-step immunoassay was prepared for quantitative

analysis of TSSPs without immobilizing antibodies. In other words, this immunoassay could be performed by mixing the samples with the antibody solution. In this solution-based one-step immunoassay, anti-TSSP antibodies were labeled with a fluorescence quencher (TQ2), and the corresponding mimotopes labeled with fluorescein were bound to the paratopes of immobilized monoclonal antibodies, as shown in Fig. 8(a). When a positive sample with the corresponding antigens was added, the pre-bound mimotopes (with fluorescence labels) were released according to the concentration of antigens in the sample. In the case of negative samples without the corresponding antigens, there was no change in fluorescence in the solution. Using this assay configuration, the concentration of food allergens could be estimated by mixing the

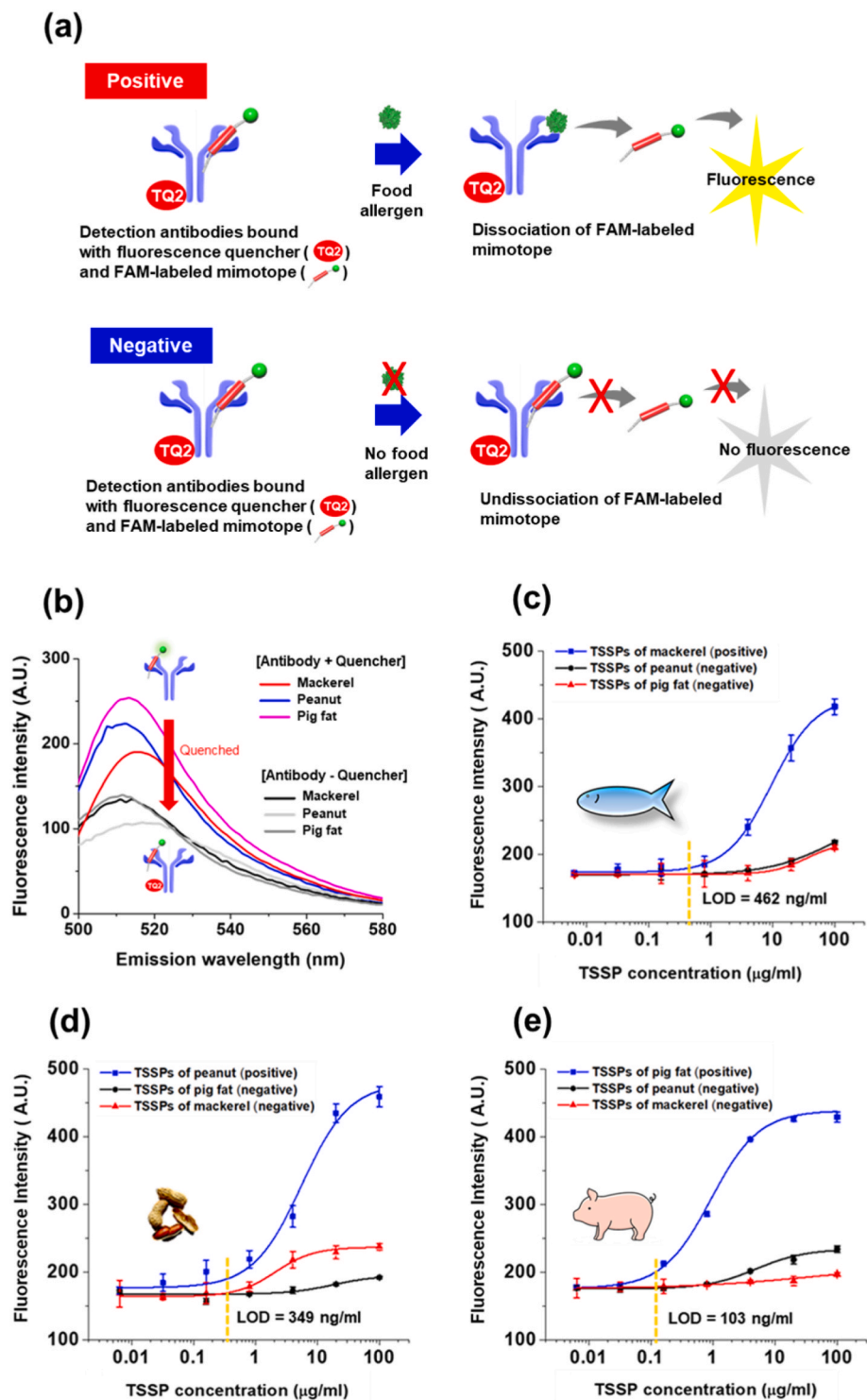


Fig. 8. One-step immunoassay based on mimotopes in solution. (a) Configuration of one-step immunoassay. (b) Fluorescence spectrum before and after labeling TQ2 quencher ($\lambda_{\text{ex}} = 492 \text{ nm}$, $\lambda_{\text{em}} = 521 \text{ nm}$) to detection antibodies. Standard curves for the one-step immunoassay of food antigens isolated from (c) mackerel, (d) peanut, (e) pig fat. The error bars in the graphs represented the standard deviation at each concentration.

samples with the antibody solution.

The quenching event through the interaction between the fluorescence of mimotope labeled with FAM ($\lambda_{\text{ex}} = 492 \text{ nm}$, $\lambda_{\text{em}} = 521 \text{ nm}$) and the labeled quencher at the detection antibodies (TQ2, $\lambda_{\text{abs, max}} = 516 \text{ nm}$) was investigated. As the first step, the fluorescence signal was measured when the mimotope were bound to the detection antibodies. And then, it was compared with the fluorescence signal from the detection antibodies labeled with the fluorescence quencher (TQ2) at

the same condition. As shown in Fig. 8(b), the fluorescence signal from the mimotope was observed to be reduced (quenched) to be 36.2% for antibodies against mackerel, 42.6% for antibodies against peanut, 48.2% for antibodies against pig fat. These results showed that the fluorescence signal of the mimotope labeled with FAM, $\lambda_{\text{ex}} = 492 \text{ nm}$, $\lambda_{\text{em}} = 521 \text{ nm}$) was effectively absorbed by the labeled quencher at the detection antibodies (TQ2, $\lambda_{\text{abs, max}} = 516 \text{ nm}$). These results represented that the one-step immunoassay based on mimotopes were

feasible in solution using the detection antibodies labeled with the fluorescence quencher (TQ2). As shown in Fig. 8(c)–(e), a one-step immunoassay of TSSPs of mackerel, peanut, and pig fat was demonstrated using the immobilized antibodies against TSSPs with mimotopes of corresponding TSSPs. In the case of the mackerel, a one-step immunoassay was possible in the TSSP concentration range of 64 ng/ml–100 µg/ml. For TSSPs of peanut and pig fat, a one-step immunoassay was possible in the TSSP concentration range of 64 ng/ml–100 µg/ml and 0.064–100 µg/ml, respectively. These results showed that one-step immunoassays for the quantitative analysis of TSSPs were possible without immobilizing antibodies to any solid support, such as microplates or microbeads.

The commercially available lateral flow immunoassay kits for peanut and pig fat were used to compare the analytical parameters with the one-step immunoassay based on mimotopes. However, the lateral flow immunoassay kit for mackerel was not commercially available. The commercial lateral flow immunoassay kits were produced to have LOD of 5 ppm for total peanut (1.3 ppm of peanut protein) and LOD of 0.5% pork meat (5 µg/ml pork protein). The test results of lateral flow immunoassay kit were compared with the one-step immunoassay based on mimotope using the same sample solution of TSSPs. As shown in Fig. 9, the lateral flow immunoassay kit for peanut was estimated to have LOD of 849 ng/ml of TSSP in comparison with 349 ng/ml of TSSP for the one-step immunoassay. The detection range of one-step immunoassay was far wider than the lateral flow immunoassay kit, and the sensitivity of one-step immunoassay was observed to be far higher at the whole concentration range, as shown in Fig. 9(a). In the case of pig fat, the LOD of the lateral flow immunoassay kit was estimated to be 3.45 µg/ml of TSSP in comparison with 103 ng/ml of TSSP for the one-step immunoassay. And, the sensitivity of one-step immunoassay for pig fat was also significantly higher than the lateral flow immunoassay kit at the whole concentration range, as shown in Fig. 9(b). Additionally, hook effect was observed for the lateral flow immunoassay kit in the range of high concentration. These results showed that the one-step immunoassay based on mimotopes could be used for more sensitive detection of food allergens with wider concentration range in comparison with the lateral flow immunoassay kits.

4. Conclusions

The F_V-antibody library was prepared from the F_V region of a heavy chain (V_H) of IgG, which was generated by site-directed mutagenesis of the CDR3 region with 11 amino acids. The diversity of autodisplayed F_V-antibody library was estimated to be 2.8×10^5 clones/library from a random selection of cultured clones, and the yield of expression was estimated to be 2.02×10^5 proteins/*E. coli* as demonstrated via densitometry. Food allergens against mackerel, peanut, and pig fat were isolated from the boiled supernatant as TSSPs, and monoclonal antibodies were obtained using the TSSPs against food allergens. For the screening of F_V-antibodies with a binding affinity toward the monoclonal antibodies against TSSPs, magnetic beads with immobilized monoclonal antibodies against TSSPs were mixed with *E. coli* with autodisplayed F_V-antibodies. For each anti-TSSP antibody against mackerel, peanut, and pig fat, one *E. coli* clone was screened as a mimotope of corresponding TSSP, and the yield of expression was estimated to be 3.41×10^5 , 2.54×10^5 , and 1.59×10^5 proteins/*E. coli* for TSSP of mackerel, peanut, and pig fat, respectively, as demonstrated via densitometry. The screened clones with autodisplayed F_V-antibodies on the outer membrane were analyzed using fluorescence-labeled anti-TSSP antibodies. The immunostaining results showed that (1) there was no specific binding of antibodies against TSSPs to the outer membrane of *E. coli*, (2) F_V-antibodies on the screened *E. coli* clone against TSSPs of peanut and pig fat had a specific binding affinity to monoclonal antibodies against the corresponding TSSPs, and (3) binding of the fluorescence-labeled antibodies against TSSPs to the screened clones occurred in the CDR3 region of F_V-antibodies of the screened clones.

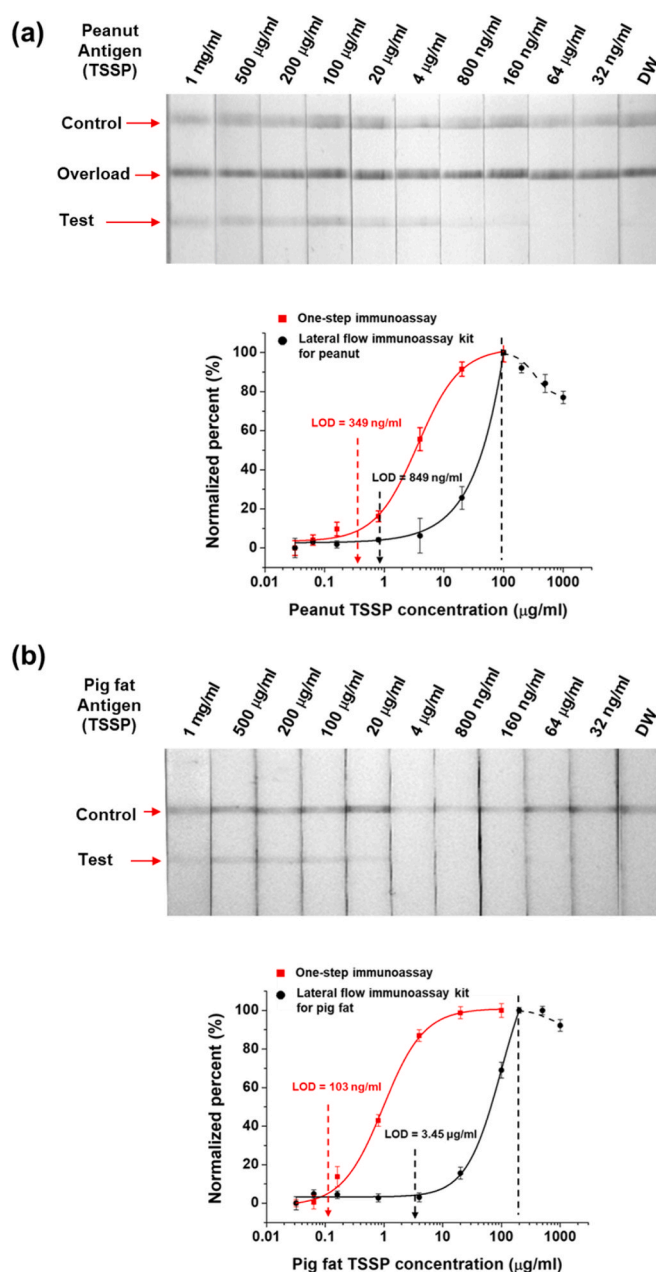


Fig. 9. Comparison of analytical parameters of the one-step immunoassay based on mimotopes with commercial lateral flow immunoassay tests for (a) peanuts and (b) pig fat using TSSPs as analytes.

These results indicated that the CDR3 regions could be used as mimotopes (mimicking peptides) of TSSPs of food allergens, and the CDR3 regions of the screened clones (11 amino acids) were synthesized as peptides. The homology of the amino acid sequences of the mimotopes was analyzed using BLAST software. The mimotope of three food allergens was analyzed to have a high amino acid homology with proteins from specific bacterial strains. Two types of one-step immunoassays of TSSPs were demonstrated using the CDR3 regions of the screened clones (11 amino acids) as a probe. For one-step immunoassay, anti-TSSP antibodies were immobilized on the microplate, and the synthetic peptides from the CDR3 regions of screened clones were bound to the paratopes of immobilized antibodies, and the quantitative analysis of TSSPs was possible using the TSSPs synthetic peptides of CDR3 regions from the screened clones. Another type of one-step immunoassay was prepared for the quantitative analysis of TSSPs just by mixing samples with the antibody solution. In this solution-based one-step immunoassay, anti-

TSSP antibodies were labeled with a fluorescence quencher (TQ2), and the corresponding mimotopes labeled with fluorescein were bound to the paratopes of the immobilized monoclonal antibodies.

CRediT authorship contribution statement

Jeong Soo Sung: Experiment, Data curation, Writing – original draft. **Ji-Hong Bong:** Experiment, Data curation. **Soo Jeong Lee:** Experiment, Data curation. **Jaeyong Jung:** Experiment, Data curation. **Min-Jung Kang:** Validation, Supervising. **Misu Lee:** Validation, Supervising. **Won-Bo Shim:** Validation, Supervising. **Joachim Jose:** Validation, Supervising. **Jae-Chul Pyun:** Supervision, Funding acquisition, Writing – original draft, review & editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bios.2022.113976>.

Abbreviations

CDRs	complementarity determining regions
FRs	frame regions
IPITG	isopropylthio- β -galactoside
PBS	phosphate buffered saline
TSSPs	thermostable soluble proteins
TQ2	Tide Quencher™ 2

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This work was supported by the National Research Foundation of Korea [NRF-2020R1A2B5B01002187, NRF-2020R1A5A101913111 and NRF-2021R1A2C209370611].

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