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Predictive selection and evaluation of appropriate functional peptides for intestinal delivery with a porous silica gel

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Bioactive peptides have a positive impact on body functions and conditions and may influence health. However, peptides are degraded by digestive enzymes, such as pepsin in the stomach when ingested orally. In order to solve this problem, we previously focused on porous silica gel and found that by using calcined silica gel, hydrophobic and negatively charged peptides could be efficiently delivered into the intestine, because peptides adsorbed on the cavity of the silica gel could be protected from enzymatic degradation. Therefore, in this study, we attempted to develop peptides whose physicochemical properties were suitable for intestinal delivery without lowering their activity. We also proposed guidelines of predictive selection of such peptides. For that purpose, we selected hypercholesterolemic peptides as a model and re-designed the peptides based on the previously reported color map, in which intestinal delivery degree was predictively depicted as contour lines. As a result, we succeeded in getting five different re-designed peptides from 1265 substituted peptide derivatives. These peptides showed a dual function of being suitable for intestinal delivery with silica gel and for disruption of bile acid micelles. The release amount of IYEYMY was 2.09 times the parent peptide, which was the highest.

[**Key words:** Peptide design; Peptide screening; Porous silica gel; Oral administration; Bile acid; Cholesterol micelle]

Bioactive peptides (BPs) have been defined as specific protein fragments that have a positive impact on body functions or conditions and may influence health (1). BPs play a significant role in human health by showing antioxidative, antimicrobial, antihypertensive, cytomodulatory, immunomodulatory, and hypercholesterolemic activities (2,3). They usually consist of 3–20 amino acid residues and these peptides are attracting attention as active ingredients of functional and health foods. However, there are various problems in developing them (4,5). One of them is enzymatic degradation. In general, peptides are degraded by digestive enzymes, such as peptidases and proteases in the stomach when ingested orally (6,7). In order to solve this problem, we focused on porous silica gel and examined the possibility of peptide degradation-resistant carriers. Porous silica gel has many useful characteristics, including a large cavity area, pH-responsive functional groups, size controllable pore, and approval as a food additive. In our previous study, we created a new type of silica gel that had approximately a 10 nm pore size, which suggests that by controlling the pore size, about 60% of the adsorbed peptides inside the silica gel were protected from pepsin (8). In addition, we examined adsorption properties of peptides on silica gel in detail and found that they could be organized by charge and hydrophobicity, and revealed that hydrophobic and negatively charged peptides could be efficiently delivered to the intestine.

In this paper, we tried intestinal delivery of BPs with a porous silica gel. For that purpose, we used hypercholesterolemic peptides as a model. In humans, cholesterol absorption occurs in the proximal jejunum of the small intestine, where both dietary cholesterol and biliary cholesterol are available for uptake from the intestinal lumen via bile acid micelles (9). Therefore, disruption of bile acid micelles could suppress intestinal cholesterol absorption, and a number of such inhibitory substances have been reported (10–14). VAWWMY, derived from soybean protein, has been investigated as a peptide. Nagaoka et al. (15) have reported that VAWWMY, which is the most hydrophobic 6-mer peptide of the glycinin A1aB1b subunit of the soybean protein, could inhibit cholesterol absorption and reduce serum cholesterol. This peptide binds to taurocholic acid, which is the micelle-forming substance, and causes inhibition of micellization. Thereafter, with VAWWMY in mind, we screened 6-mer peptides replacing the amino acids and discovered 10 new bile acid-binding peptides (16,17).

We previously showed that calcining silica gel could deliver hydrophobic and negatively charged peptides to the intestine efficiently (8). In the current study, we tried to develop new peptides that have a dual function of being suitable for intestinal delivery on silica gel and for the disruption of bile acid micelles. For that purpose, we performed residue substitution for 11 bile acid-binding peptides, which were listed by our group and Nagaoka's group (15–17). To screen the dual functional peptides from a huge number of substituted ones, we proposed predictive selection

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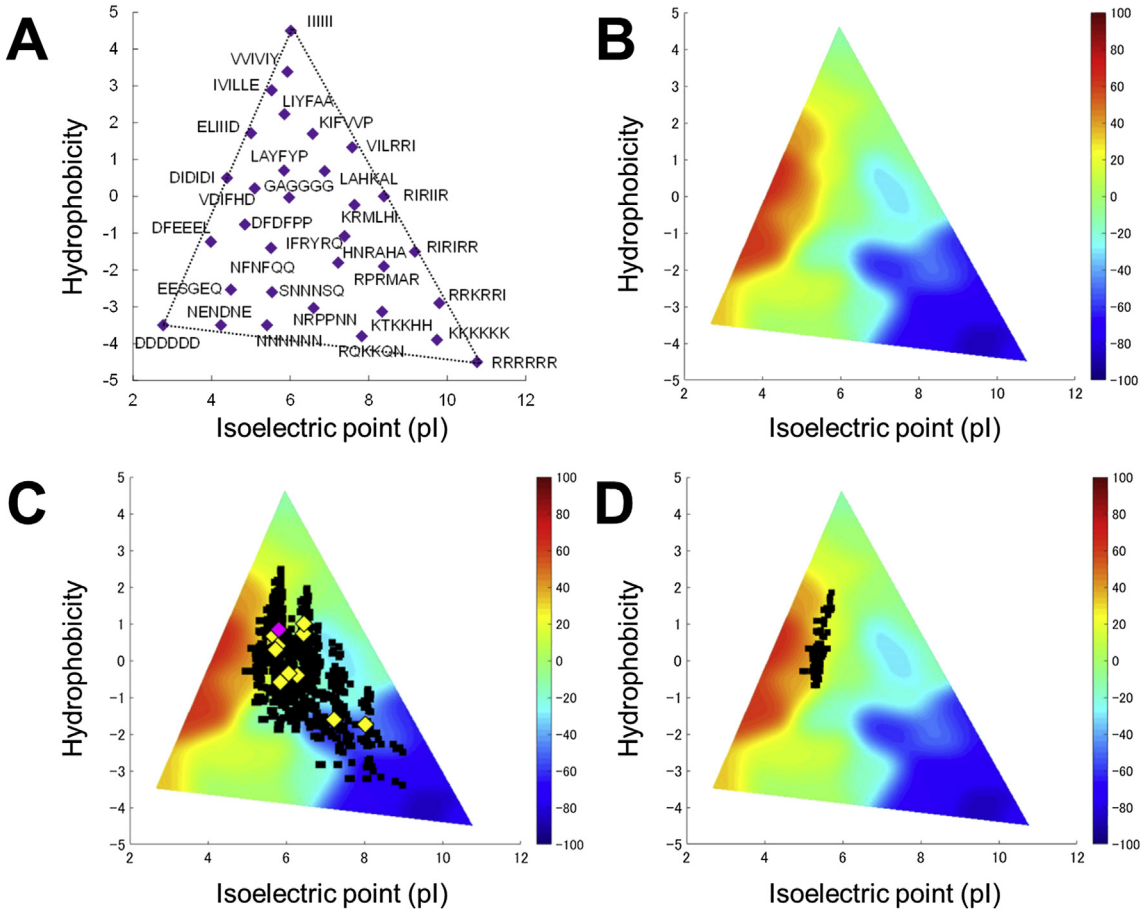

FIG. 1. Predictive selection of dual-functional peptides. (A) Scatter diagram classified by hydrophobicity versus pI. Purple diamonds denote representative hexa-peptides. (B) Color map based on the results of panel A. (C) A scatter diagram of 1265 substituted peptides. All 1265 candidate peptides made from single residue substitutions are plotted on the color map. Pink diamond denotes VAWWMY that was the parent peptide. The 10 yellow diamonds denote other bile acid-high binding peptides that were discovered previously. Black squares denote all candidate peptides. (D) The 78 selected peptides whose score values were 20 or more.

based on the physicochemical properties suitable for silica gel delivery without lowering the activity.

MATERIALS AND METHODS

Materials Porous silica gel, SMB-100-5 was supplied by Fuji Silysia Chemical Ltd., Aichi, Japan. Heat-treated silica gel was created from SMB-100-5 by calcining at 600 °C for 2 h under an air atmosphere (FG31, Yamato Scientific Co., Ltd., Tokyo, Japan).

Peptide synthesis for the evaluation of adsorption properties of silica Peptide arrays were synthesized using a cellulose membrane and a spot synthesizer (ASP222, Intervis, Cologne, Germany) as previously described (18). After punching, each of the resulting peptide-containing disks (peptide spots) was placed in a single well of a 96-well plate filter (MSRLN0410; Merck Millipore, Burlington, MA, USA) and 180 μL of buffer solution [in the pH 2.1 experiments, 100 mM phosphate buffer, and in the pH 7.4 experiments, phosphate buffered saline (PBS)] was added. After 1 h incubation at room temperature, the solution containing peptides was released from the disk and filtered into a 96-well plate by vacuum filtration. Each filtrate was used for peptide adsorption experiment. Porous silica gel was suspended in a buffer solution at 100 mg/mL. The peptide solution that was released from the peptide disk as described above was utilized. A 150 μL of the peptide solution and a 50 μL of the suspension (50 μL of buffer solution was used as reference) were mixed and shaken with vortex mixer for about 30 s. After being centrifuged at 9300 ×*g* for 1 min, the amount of adsorbed peptide was determined by measuring the amount of peptide remaining in the supernatant. The amount of peptide was quantified by fluorimetric assay (19). For the pH 7.4 experiments, 10 μL of fluorescamine (5 mg/mL in acetone) was added to 150 μL aliquot of the supernatant in a 96-well plate, and the fluorescence intensity was measured with excitation at 355 nm and emission at 460 nm (Fluoroskan Ascent Microplate; Thermo Fisher Scientific, Waltham, MA, USA). For the pH 2.1 experiments, before fluorimetric assay, the supernatant pH was adjusted to 7.4 (200 μL of 0.1 N NaOH was added to 150 μL of the supernatant). After that, 150 μL of solution was utilized for fluorimetric assay. Since PYWFDM and PYWFEM have secondary amino groups, fluorescence could not be detected. Therefore, we quantified them using HPLC (220 nm). To evaluate the intestinal delivery, we defined a score value given by the equation below (8).

$$\text{Score value} = \text{pH 2.1 adsorption amount} - \text{pH 7.4 adsorption amount} \quad (1)$$

All assays were repeated thrice and data are presented as mean values ± standard deviation (SD).

Scatter diagrams and color maps To generate the comprehensive peptide scatter diagram, we chose two indices; hydrophobicity (20) and isoelectric point (21). For example, all tripeptides in the scatter diagram were plotted on the basis of the following equations:

$$\text{Hydrophobicity} = (X_{i1} + X_{i2} + X_{i3})/3 \quad (2)$$

$$\text{Isoelectric point (pI)} = (Y_{i1} + Y_{i2} + Y_{i3})/3 \quad (3)$$

where X_i and Y_i are the hydrophobicity value and pI value of amino acids in the peptide i, respectively. Subscript 1, 2, and 3 indicate the 1st, 2nd, and 3rd amino acids from the N-terminal of peptide i, respectively. Thus, X_i and Y_i indicate the average hydrophobicity and pI of the peptide i, respectively. We created 3D color maps with MATLAB based on the score values, hydrophobicity, and pI of the peptides.

First screening of peptides All candidate peptides were plotted on the color map and coordinates were read. In this study, we selected only those whose values were 20 or more.

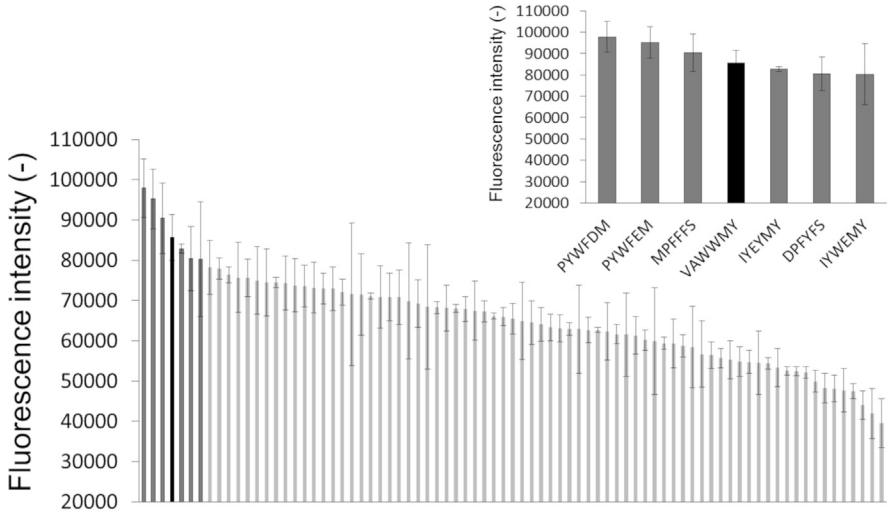

FIG. 2. Fluorescence intensity of taurocholic acid binding to the 78 peptides. Fluorescence intensities of high binding peptides are highlighted at the upper-right. Top six peptides (not including VAWWMY) had almost the same binding activity as VAWWMY (n = 3).

Screening of bile acid-binding peptides in peptide arrays Screening of bile acid-binding peptides were conducted by the method used by Takeshita et al. (16) with some modifications. Peptide arrays were soaked in 1% BSA and PBS for 12 h at 4 °C for blocking. After blocking, the arrays were incubated with taurocholate (T4009; Sigma–Aldrich, St. Louis, MO, USA) and PBS at a final concentration of 10 μg/mL for 1 h at 37 °C with slow agitation. After washing with PBS, the arrays were incubated with anti-cholic acid antibody (FKA-502; Cosmo Bio, Tokyo, Japan) in PBS containing 0.25% BSA for 1 h at 37 °C, followed by a wash with Tris-buffered saline containing 0.05% Tween-20. Peptide arrays were next incubated with 2 μg/mL anti-rabbit IgG conjugated with Alexa 488 (ab150077; Abcam, Cambridge, UK) in PBS for 1 h at 37 °C. After washing with Tris-buffered saline (TBS) at 37 °C, the peptide spots were fluorescently detected with a fluorescent imager (Typhoon FLA-9500; GE Healthcare Japan Life Sciences, Tokyo, Japan). The scanned images were quantified using Image Quant TL (GE Healthcare Japan Life Sciences) and the fluorescence intensity of each spot was background adjusted. Average of triplicate fluorescence intensities (on the primary antibody-treated array) was subtracted from the peptide array treated only with secondary antibodies containing the same peptide sequence.

Peptide adsorption and desorption experiment Peptide adsorption and desorption experiments were conducted by the method used by Imai et al. (8). A 150 μL of silica suspension (25 mg/mL) and a 150 μL of peptide solution (0.5 mM) were prepared and shaken with vortex mixer for about 30 s, and left to equilibrate for 5 min at room temperature. After being centrifuged (at 9300 ×*g* for 1 min) to separate the supernatant and silica gel, 300 μL of phosphate buffer (pH 2.1) was added to the silica gel and shaken vigorously, and left to equilibrate for 5 min at room temperature. This was the acidic release step. Next, the mixture was separated in the same way and 300 μL of PBS (pH 7.4) was added to the silica gel and shaken vigorously, and left to equilibrate for 5 min at room temperature. This was the neutral release step. The peptide release amounts were quantified by fluorimetric assay described above and the release of PYWFDM and PYWFEM was quantified by HPLC.

Cholesterol micelle preparation and determination of disrupting micelles by peptides Cholesterol micellar solubility with peptides was measured *in vitro* with reference to Raederstorff et al. (10) and Nagaoka et al. (22) with some modifications. The mixed micelles were prepared by sonication (30 min) of PBS containing 6.6 mmol/L sodium taurocholate, 0.5 mmol/L cholesterol, 1 mmol/L oleic acid, 0.5 mmol/L monoolein, and 0.6 mmol/L phosphatidylcholine at pH 7.4. The lipids were dissolved in methanol and dried before adding the PBS. The micellar solution was kept at 37 °C for more than 24 h. Peptides were then added to this micellar solution and dissolved by sonication and incubated at 37 °C for 24 h. When micelles disrupted, intermicellar cholesterol precipitated. In order to separate the precipitated cholesterol, the solution was centrifuged at 10,000 ×*g* for 20 min and the supernatant was filtered through a 0.22 μm filter. Intermiceller cholesterol in the supernatant was determined using the cholesterol kit (439-17501; Wako, Osaka, Japan). Then, 0.5 mL of filtrate was added to 3 mL of kit solution and absorbance at 600 nm was measured.

Statistical analysis Data are presented as mean values and standard deviation (SD), and Student's *t* test was used for evaluating statistical significance for comparison. A value less than 0.05 ($p < 0.05$) versus VAWWMY indicated statistical significance ($^{**}p < 0.01$, $^{*}p < 0.05$).

RESULTS AND DISCUSSION

Drawing a hexa-peptide color map and peptide screening In our previous report, we prepared color maps in which degree of intestinal delivery were predicted and depicted as contour lines. In these maps, score values of 32 tri-, penta-, and hepta- peptides were depicted. In the present study, a new color map was drawn with hexa-peptide library in the same way. We selected 32 kinds of peptides with varying hydrophobicity and pI (Fig. 1A), conducted adsorption experiments, and calculated score values (Table S1). Based on these results, we drew a hexa-peptide color map (Fig. 1B). The map was classified by hydrophobicity versus pI and high score values were upper left area. This result indicated that high hydrophobicity and anionic peptides were suitable for oral intestinal delivery using the heat-treated silica gel. This tendency was also observed in our previous report (8).

Nagaoka 's group and our group (15–17) discovered 11 kinds of bile acid-binding peptides (VAWWMY, PWWWMY, IPWYFY, VIWWFK, IYWYMY, PVRWKK, RPYFYK, LPRYIE, PYWFHM, MPFYFS, and KVWYMV). We focused on these peptides as models to investigate the delivery efficiency and plotted these peptides on the color map (VAWWMY, which is the parent peptide was plotted as a pink diamond, and other peptides were plotted as yellow diamonds) (Fig. 1C), and read the score values (Table S2). Unfortunately, all 11 peptides had low score values, which meant none of the peptides were suitable for oral delivery with silica gel. To solve this problem, we tried to substitute individual amino acids while keeping the functionality of binding with bile acids. We screened peptides made from all single residue substitutions (1265 such peptides were depicted with black squares).

We plotted 1265 different candidate peptides on the color map and searched for those with higher score values (Fig. 1C). The score value of parent peptide, VAWWMY was 14.36. We found 260 peptides with a value of 14.36 or more and 78 peptides with a value of 20 or more. We selected these 78 peptides and used them for the following experiments (Fig. 1D, Table S3).

Bile acid-binding assay Next, we evaluated the intestinal delivery and bile acid-binding activity of 78 screened peptides *in vitro*. At first, we synthesized the 78 peptides and investigated the binding activity. We found that six of them retained almost the same binding activity as that of VAWWMY (Fig. 2). It seemed

TABLE 1. The bile acid-binding activities and score values of six peptides and VAWWMY.

Number	Sequence	Bile acid binding activity	Score value
1	PYWFDM	1.14	1.56
2	PYWFEM	1.11	2.25
3	MPFFFS	1.06	0.32
4	IYEYMY	0.97	4.90
5	DPFYFS	0.94	2.77
6	IYWEMY	0.94	3.32
Parent peptide	VAWWMY	1.00	1.00

All values were standardized with respect to the parent peptide (VAWWMY).

that the overall binding activity of the 78 peptides was considerably decreased by the substitution. This might have been because the residues that are important for binding to bile acids were substituted.

There are many studies about the interaction of proteins and bile acids. Gough et al. (23) examined the interaction between nisin and bile acids and revealed that positively charged regions of nisin are responsible for its initial interaction with the negatively charged bile acids. Other reports revealed that hydrophobic amino acids, especially the aromatic ones, interact with bile acid micelles (23–26). These reports indicate that positively charged and aromatic amino acids could be important for binding.

In our screening, since positively charged peptides were not chosen, it was considered that the overall binding activity has declined. In this screening, we discovered six peptides, namely PYWFDM, PYWFEM, MPFFFS, IYEYMY, DPFYFS, and IYWEMY, which retained almost the same binding activity as VAWWMY. Half of them were composed of aromatic amino acids, which seemed to be the reason why these sequences have a high bile acid-binding activity. Next, we evaluated intestinal delivery properties of these six peptides.

Evaluation of score values We synthesized six peptides and evaluated their score values (Table 1). It was found that five out of the six peptides had score values greater than that of VAWWMY. This means that we succeeded in screening the peptides with a potential for intestinal delivery by silica gel while keeping the bile acid-binding activity. The highest score was achieved by IYEYMY, which was 4.90 times the parent peptide. On the other hand, MPFFFS was expected to have twice the score of VAWWMY, but it was a mere 0.32 times. The predicted and the measured data were investigated (Table S4), and it was found that a prediction error occurred in the range of −16 to +24. Whereas, the average predicted value of six peptides was 27.4 and the predicted value was within −100 and 100. This may be due to the inaccuracy of the color map. In our color map, we acquired data with 32 representative peptides, and in the region other than that of the data points, we predicted the scores. The accuracy probably reduced with distance from the data points. Another reason was the indices of pI and hydrophobicity of the whole peptide that we employed to draw the color map. By using these two indices, we could grasp a rough tendency, but it was impossible to represent all the properties of the peptides. In order to make a more precise prediction, other indicators should be considered.

Although the prediction error was not small, it was much more valuable that five of six peptides showed a high score value. Therefore, it was concluded that this color map is useful in depicting the behavior of all the peptides and designing guidelines. To achieve more precision in predictive selection, more data points need to be acquired and a more accurate color map should be created.

Evaluation of release of peptides Using the score value proposed by us, we could screen the peptides suited for the intestinal delivery of peptides by silica gel with a high throughput. If the score value was high, the intestinal delivery amount tended to be high (8). Therefore, in order to confirm whether the relationship between score values and the release amount had a positive correlation, the release amount was measured. According to the tendency of the score value, only MPFFFS had a lower release amount, while the other peptides had higher release amounts compared to VAWWMY (Fig. 3). IYEYMY, had the highest release amount of 2.96 ± 0.12 mg/g which was up to 2.09 times that of VAWWMY (1.41 ± 0.06).

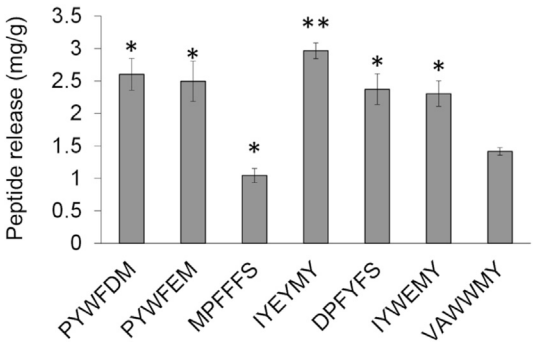

FIG. 3. The release amount of the six peptides and VAWWMY under pH 7 (n = 3). $^{**}p < 0.01$, $^{*}p < 0.05$ versus VAWWMY.

Cholesterol solubility of micelles Bile acid-binding peptides were expected to inhibit cholesterol absorption by disrupting the bile acid micelle. We evaluated the disruption of the bile acid micelle by the selected peptides. The selected peptides were added to the micellar solution and cholesterol solubility was measured (Fig. 4). At the peptide concentration 10 mg/mL, all five peptides disrupted the micelles completely. We also confirmed that VAWWMY and cholestyramine, a strong ion exchange resin that binds bile acid, showed disruption activity, and casein hydrolysates did not show disruption as reported previously (15,17). We carried out the same experiment with a concentration of 5 mg/mL. Among the five peptides, only IYWEMY showed a strong micelle-disruption activity.

Next, we investigated the relationship between the concentration of IYWEMY and the solubility of micelles (Fig. 5). Although VAWWMY and cholestyramine disrupt micelles in a concentration-dependent manner, IYWEMY dramatically changed the trend of disruption between 3 mg/mL and 4 mg/mL, which indicated that IYWEMY could disrupt micelles via a different mechanism.

In our screening, we discovered the IYWEMY peptide. When the peptide delivery amount was 1–3 mg, VAWWMY was superior because IYWEMY showed no disruption activity at all ($p < 0.05$). However, when the amount was 5 mg or more, both peptides showed almost a 100% disruption activity. In order to transport 5 mg, IYWEMY needed 2.17 g of silica gel and VAWWMY needed 3.53 g theoretically, which means we can save 39% of the silica gel and obtain the same effect. Hence, using this design scheme, we succeeded in designing peptides that could deliver efficiently using the silica gel. This scheme is expected to be useful not only to hypercholesterolemic peptides, but also to other functional peptides.

Screening peptides that have higher cholesterol micelle disruption activity is valid scheme because of saving the amount of peptides and that have higher score value is also valid because of delivering large quantities. In this study, we focused on the latter. For screening peptides that have cholesterol micelle disrupting activity, we performed bile acid binding assay. As shown in Figs. 2 and 4, all screened peptides have disruption activity. This result indicated the validity of this screening. However, the top bile acid binding peptide, PYWFDM did not have the highest cholesterol micelle disruption activity. This result indicated that there are

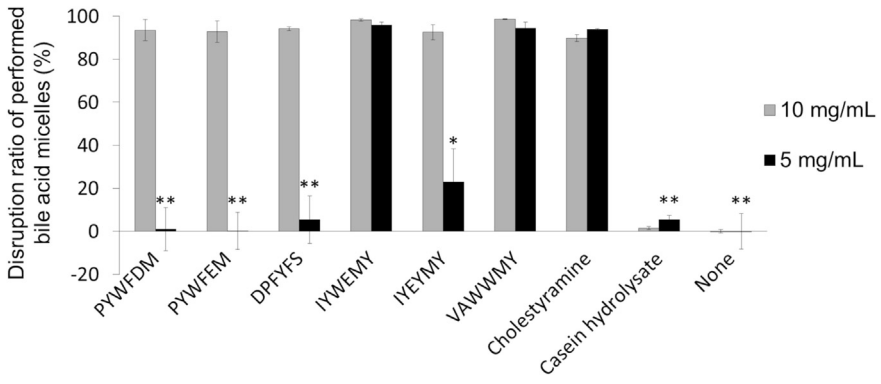

FIG. 4. Disruption ratio of bile acid micelles by bile acid-binding peptides. Cholestyramine and VAWWMY were used as positive control and casein hydrolysates as a negative control. Shaded bars represent under 10 mg/mL and closed bars represent under 5 mg/mL. None means no peptides were added (n = 3). $^{**}p < 0.01$, $^{*}p < 0.05$ versus VAWWMY.

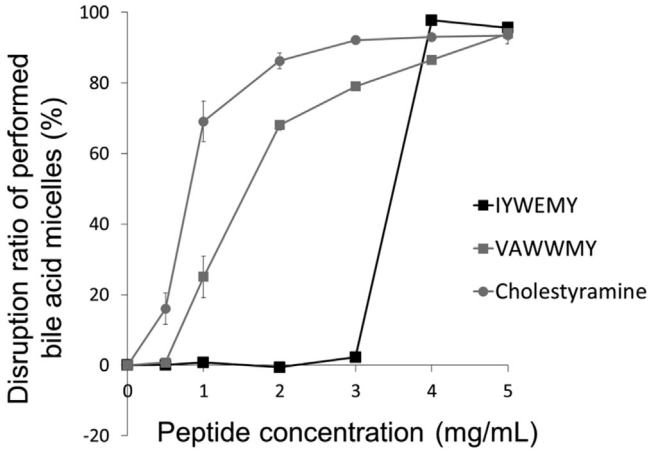

FIG. 5. Dose response of IYWEMY peptide on micelle disruption. VAWWMY and cholestyramine were used as positive controls (gray line).

important factors other than bile acid binding activity. Now we do not know what it is and would elucidate these factors.

We are in the process of further improving our design guidelines for practical application. BPs are mainly obtained in protein hydrolysates. If the designed peptide sequence does not exist in natural storage proteins, it is difficult to utilize them as functional foods. We are now working on developing a more practical screening program that can screen the database of natural protein sequences. In addition, we would elucidate a more detailed disruption mechanism of newly discovered IYWEMY and conduct *in vivo* experiments.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.01.001.

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