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Screening of inhibitors for influenza A virus using high-performance affinity chromatography and combinatorial peptide libraries

Rui Zhao[a,*], Canliang Fang[a,b], Xiao Yu[a], Yang Liu[a,b], Jia Luo[a,b], Dihua Shangguan[a], Shaoxiang Xiong[a], Tiansheng Su[a], Guoquan Liu[a,**]

[a] *Center for Molecular Science, Institute of Chemistry, Chinese Academy of Sciences, Zhong Guan Cun, Beijing 100080, China*
[b] *Graduate School of Chinese Academy of Sciences, Beijing 100039, China*

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Abstract

The affinity inhibitor of fusion peptide of influenza A virus has been studied using a combination of high-performance affinity chromatography (HPAC) and combinatorial peptide libraries. Fusion peptide (FP) (1–11) of influenza A virus was used as the affinity ligand and immobilized onto the poly(glycidyl methacrylate) (PGMA) beads. Positional scanning peptide libraries based on antisense peptide strategy and extended peptide libraries were designed and synthesized. The screening was carried out at acidic pH (5.5) in order to imitate the environment of virus fusion. A hendecapeptide FHRKKGRGKHK was identified to have a strong affinity to the FP (1–11). The dissociation constant of the complex of the hendecapeptide and the FP (1–11) is 3.10×10^{-6} mol l^{-1} in a physiological buffer condition. The polypeptide has a fairly inhibitory effect on three different strains of influenza A virus H1N1 subtype.

Keywords: High-performance affinity chromatography; Combinatorial peptide library; Inhibitor; Influenza A virus; Screening

1. Introduction

Influenza is a common and widespread disease and seriously endangers human and animal's health and life. The influenza pandemic of 1918–1919 has been cited as the most devastating epidemic in recorded world history [1]. Influenza also results in tremendous economic costs both from admissions to hospital and loss of productivity [2]. However, influenza is very difficult to be prevented and treated because of frequent mutation of the virus. Vaccination provides limited protection due to the difficulty in prediction of predominant virus subtype in the coming season. Currently, the M2 ion channel blockers, amantadine and rimantadine [3,4] as well as the neuraminidase (NA) inhibitors, zanamivir [4,5] and oseltamivir [4,6] are commercially available. Disadvantages of the M2 channel blockers are efficacy against influenza A virus only and the relative rapid development of resistance [7]. The NA inhibitor drugs are active against both influenza A and B viruses but expensive for people in developing countries. So, scientists are still searching for new drug candidates for treatment of influenza infection.

In the infection cycle of influenza virus, hemagglutinin (HA) [8] firstly mediates the binding of the virus particles to the host cell surface. A dramatically conformational change of HA is triggered at the low-pH (5.0–5.5) environment so as to the hydrophobic fusion peptides (FPs) at N-terminal of HA2 are exposed from their buried position [9] and inserted into the target membrane, which results in the further fusion of viral and endosomal membranes [10]. Clearly, blockage of the fusion step is one of the ideal targets for designing inhibitors for this type of viruses. Because the sequences of the influenza virus FPs are highly conserved, it is reasonable

* Corresponding author. Tel.: +86 10 62557910; fax: +86 10 62559373.
** Co-corresponding author. Tel.: +86 10 62564817; fax: +86 10 62559373.
E-mail addresses: zhaorui@iccas.ac.cn (R. Zhao), liugq@iccas.ac.cn (G. Liu).

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to assume that compounds which tightly bind to the fusion peptide could interrupt the virus infusion step and may be potential inhibitors of influenza virus.

Affinity chromatography [11] has been widely used in protein separation and purification [12] as well as proteomics [13] and high throughput screening [14] recently. Combinatorial peptide chemistry [15,16] has become a popular tool for preparing collections of peptides with different sequences. The combination of affinity chromatography and peptide libraries has been successfully used to find inhibitors and ligands for different targets [17]. For example, a highly selective inhibitor of human betine homocysteine *S*-methyltransferase [18] was recently identified by this combination method.

In the present study, we developed a novel method for searching potential inhibitors of influenza A virus. The influenza FP (1–11) that consists of the first 11 N-terminal amino acid residues of HA2 was used as the affinity ligand. Positional scanning peptide libraries were designed based on the antisense peptide identified in our previous study [19]. A hexapeptide was identified to have high affinity to the FP (1–11) and used as the leading sequence for designing extended peptide libraries. The dissociation constants were measured by frontal affinity chromatography and the anti-influenza virus activity was also assayed.

2. Experimental

2.1. Chemicals and samples

9-Fluorenylmethoxycarbonyl (FMOC)-derivatized amino acids, FMOC-AA-Wang Resin, *N*,*N*′-dicyclohexylcarbodiimide (DCC) and *N*-hydroxybenzotriazole (HOBt) were purchased from Siam (USA). Trifluoroacetic acid (TFA) and ethanedithiol were obtained from Sigma (USA). Epichlorohydrin was from Jinda Fine Chemical Factory (China). Acetonitrile (HPLC grade) was from Fisher (USA). Piperidine, dichloromethane (DCM), *N*,*N*′-dimethylformamide (DMF) were of analytical grade. Glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA) were purchased from Suzhou Anli Chemical Factory (Suzhou, China). 2,2′-Azobis(2-isobutyronitrile) (AIBN) was from Beijing Chemical Factory (Beijing, China). Poly(vinyl pyroolidone) (PVP) K-30 was got from Chinese Medical Corporation (Beijing, China). Poly(vinyl alcohol) (PVA) 17-88 was purchased from Beijing Organic Chemical Plant (Beijing, China). Sodium dodecyl sulphate (SDS) was from Shanghai No. 1 Chemical Reagent Factory (Shanghai, China). Triple distilled water was used in all aqueous buffer preparation.

2.2. Solid-phase synthesis of peptide libraries

The FMOC strategy [20] of solid-phase peptide synthesis was used for peptide syntheses. The syntheses were carried out manually. FMOC-AA-Wang Resin was used as the starting material. The elongation cycles for peptide synthesis on the Wang Resin were performed in the presence of DCC and HOBt. The following steps in the synthesis of the FP (1–11), the six positional scanning peptide libraries and the extended peptide libraries were carried out as described in our previous work [19]. The six special positional scanning peptide libraries are YRSKQX, YRSKXA, YRSXQA, YRXKQA, YXSKQA, XRSKQA, where X represents a mixture of 18 genetically coded amino acids except for cysteine and tryptophan. The extended peptide libraries can be described as sequences of X-G-R-G-K-H-K, X-O_1-G-R-G-K-H-K, X-O_2-O_1-G-R-G-K-H-K, X-O_3-O_2-O_1-G-R-G-K-H-K, X-O_4-O_3-O_2-O_1-G-R-G-K-H-K, where O represents a defined amino acid residue and X represents the same as that in the special positional scanning peptide libraries. In the preparation of these peptide libraries, a mixture of protected amino acids with a predetermined ratio was used [21].

2.3. Preparation of monodispersed, non-porous and cross-linked PGMA beads

2.3.1. Synthesis of PGMA seeds

The poly(glycidyl methacrylate) (PGMA) seeds were firstly prepared by dispersion polymerization method as described previously in our lab [22] and other literature [23]. Briefly, GMA, AIBN and PVP were used as monomer, initiator and steric stabilizer, respectively. The recipe is: 2.0 g GMA, 0.06 g AIBN, 0.2 g PVP and 17.74 g the mixture of ethanol and toluene (9.5:0.5, v/v). GMA, AIBN and PVP were dissolved in the mixed solution of ethanol and toluene in a 100 ml flask. The mixture was bubbled through with nitrogen for 20 min to remove the dissolved oxygen and the stoppered flask was shaken (100 rpm) in a glycerin-bath at 70 °C for 24 h. The resultant seeds were washed by extensive centrifugation cycles with absolute ethanol (20 ml each, three times) and vacuum-dried with an oil-pump for 1 h. The PGMA seeds were used in the following step in order to get the final products.

2.3.2. Synthesis of monodispersed, non-porous and cross-linked PGMA beads

Monodispersed, non-porous and cross-linked PGMA beads were secondly prepared by one-step swelling and polymerization method [24,25]. Briefly, EDMA was used as cross-linker in this experiment. The above-obtained PGMA seeds (0.5 g) were added in an aqueous solution containing PVA (0.4 g), SDS (0.04 g) and distilled water (40 ml) to form a suspension. The mixture of GMA (3.0 g), EDMA (2.0 g) and AIBN (0.1 g) in an aqueous solution containing PVA (0.6 g), SDS (0.06 g) and distilled water (60 ml) was emulsified by sonication for about 1 min. Sonication to form emulsified mixture was performed with an ultrasonic homogenizer containing a microtip probe (Ultrasonic homogenizer 4710 series, Ti-horn 20 kHz, Cole-Parmer, USA). The emulsified mixture was dropwise added into the suspension during magnetic stir at 100 rpm in 2 h, and then the suspension-based mixture was continued to stir at room temperature for about

6 h until the emulsified small droplets were absorbed completely by the PGMA seeds, which can be verified by optical microscope (Olympus BH-2, Japan, ocular ×10, objective ×100). The swollen particles were polymerized on a magnetic stirrer with 100 rpm at 70 °C for 24 h. The obtained copolymer beads were washed on a fritted glass funnel (filter pore size of 4 μm) with water (40 ml each, four times) and ethanol (40 ml each, three times) and vacuum-dried with an oil-pump for 1 h. The morphology of the resultant copolymer beads was analyzed by scanning electron microscope (SEM, Hitachi S-530, Japan). The epoxy groups on the surface of the beads were measured by Keen's method [26].

2.4. Preparation of high-performance affinity columns

Three grams of PGMA beads in 25 ml of 0.2 M H_2SO_4 were refluxed for 8 h. After washed on a fritted glass funnel (filter pore size of 4 μm) with water to neutral, 20 ml of 2% KOH was added to the PGMA beads and the suspension was sonicated using an ultrasonic cleaner for 10 min. After settling for 2 h, the supernatant was removed and 6 ml of epichlorohydrin was added. The reaction mixture was magnetically stirred with moderate speed at 50 °C for 1.5 h in a water bath. The unreacted epichlorohydrin was removed by extensive washing on a fritted glass funnel (filter pore size of 4 μm) with distilled water (30 ml each, six times). The resulted epichlorohydrin–PGMA beads were dried with an oil-pump for 2 h at room temperature. The content of epoxy groups on the surface of the epichlorohydrin–PGMA beads was measured using Keen's method [26].

A 20.4 mg of the FP (1–11) dissolved in a solution of 1 ml of acetonitrile and 9 ml of sodium carbonate (200 mmol l^{-1}) was mixed with 1.2 g of the epichlorohydrin–PGMA beads. The mixture was then incubated at 50 °C for 100 h on an incubator shaker. The content of the FP (1–11) immobilized on the PGMA beads was analyzed using RP-HPLC by measuring the concentration of the peptide in the supernatant. The stationary phase for control column was prepared using the same method without addition of the FP (1–11). The prepared FP (1–11)–PGMA and the control material were slurry-packed into stainless steel columns (70 mm × 4.0 mm i.d.).

2.5. Chromatographic conditions for affinity screening

A high-performance affinity chromatography (HPAC) system consisted of a Dionex gradient pump (USA), a Kratos Spectroflow 757 UV–vis detector (USA), a Rheodyne 7125 sample injection valve (USA), and a WDL-95 HPLC Workstation (China) was used. The affinity chromatographic experiments were performed using a stepwise mode with a flow rate of 0.5 ml min^{-1} at room temperature. Elution was monitored at 220 nm. The solutions of peptide libraries were prepared with triple distilled water at a concentration of 10 mg ml^{-1}. The binding and elution buffers were 10 mmol l^{-1} phosphate buffer or phosphate buffer containing different concentrations of NaCl at pH 5.5. Eluted peptide fractions were collected for further RP-HPLC and mass spectroscopic (MS) analysis.

2.6. Analysis and characterization of peptides

A RP-HPLC system consisted of a Shimadzu LC-10ATvp pump (Japan), a Shimadzu DGU-14A degasser (Japan), a Shimadzu SPD-10A UV–vis detector (Japan), a Rheodyne 7725i sample injection valve (USA) and a WDL-95 chromatographic work station (China) was used for the analysis of peptides. The synthetic peptide libraries and peptide fractions collected from the affinity chromatographic column were analyzed on a Dikma Diamond C_{18} column (250 mm × 4.6 mm i.d.) (China) with a flow rate of 1.0 ml min^{-1} using a gradient elution of aqueous acetonitrile containing 0.1% TFA. The wavelength of detection was 220 nm. The peptides were identified by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS, Bruker Daltonics, USA) and Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS, Bruker Daltonics, USA).

2.7. Determination of the dissociation constant of the complex of immobilized FP (1–11) and screened hendecapeptide

The dissociation constants of the complex of the synthetic peptides and FP (1–11) were measured by frontal affinity chromatography [27,28]. The FP (1–11) immobilized column was equilibrated with 10 mmol l^{-1} phosphate buffer plus 150 mmol l^{-1} NaCl. The hendecapeptide solutions (dissolved in the same buffer) with different concentrations ranging from 2.07×10^{-5} to 5.94×10^{-5} mol l^{-1} were introduced to the column, respectively. The absorbance of the effluent was monitored at 280 nm. The loading was continued in a frontal mode until the absorbance of the effluent reached a plateau, at which the concentration of the effluent was equal to the initial concentration of the applied peptide. The variation of the elution volume $\bar{V}$ with the concentration of peptide $[P]_0$ was evaluated by the equation

$$\frac{1}{\bar{V} - V_0} = \frac{K_d}{M_T} + \frac{1}{M_T}[P]_0$$

where $\bar{V}$ is the elution volume at which the affinity matrix is half-saturated (l) and V_0 is the void volume (l), M_T is the total amount of immobilized FP (1–11) (mol), $[P]_0$ is the initial concentration of hendecapeptide solution (mol l^{-1}) and K_d is the dissociation constant of the complex of immobilized FP and hendecapeptide (mol l^{-1}). From the plot of $1/(\bar{V} - V_0)$ versus $[P]_0$, K_d can be calculated by the ratio of intercept/slope.

2.8. Inhibition activity of the polypeptide on influenza A virus

Anti-influenza virus activity of the hendecapeptide identified from the affinity chromatography screening was

measured by neutralization test [29]. The three H1N1 strains of influenza virus used were A/HUFANG/7/1999, A/CNIC/143/2001 and A/GUIZHOU/259/2000. Briefly, the hendecapeptide samples with the concentration of 2.0 mg ml^{-1} were prepared by dissolving in serum-free minimal essential medium (MEM). The virus was diluted with serum-free MEM in 10x series (10^{-1} to 10^{-5}). Then, the peptide solution was mixed with differently diluted virus solution and the mixture was incubated at 4 °C for 2 h, respectively. The confluent growing Madin–Darby canine kidney (MDCK) cells were washed with Hanks' solution twice, inoculated with the peptide treated virus samples and incubated at 35 °C for 48 h. The progression of viral-induced cytopathic effects (CPE) was observed and the anti-virus activity was calculated by hemagglutination of the supernatant of peptide–virus incubated MDCK cell cultures. The controls were carried out using the same procedures without addition of peptide sample. The experiment with each dilution of the virus was repeated twice.

3. Results and discussion

3.1. Design of special positional scanning peptide libraries

Normal positional scanning peptide libraries [15] of hexapeptide consist of six positional libraries, each of which has a single defined amino acid at one position and a mixture of amino acids at each of the other five positions. They can be represented as O_1XXXXX, XO_2XXXX, . . ., $XXXXXO_6$, where O represents a defined amino acid residue and X represents a mixture of genetically coded amino acids. If all 20 genetically coded amino acids are used, each peptide sublibrary will contain 20^5 individual sequences, and 120 sublibraries (20×6) will be produced and screened for seeking high affinity compounds. This process would be very expensive and time-consuming.

In our previous work [19], YRSKQA, the antisense peptide of the FP (1–11), was found to possess the highest affinity to the FP (1–11) among the antisense peptides tested. In the present work, YRSKQA was used as the lead compound. Six special positional scanning peptide sub-libraries based on antisense peptide strategy were designed as described in Fig. 1. In each sub-library, amino acid residues at five of the six positions in the hexapeptide were kept as same as in the parent peptide and only one position was changed to the mixture of 18 natural amino acids (cysteine and tryptophan were excluded). Each sub-library contained 18 different sequences, and six sub-libraries contained 108 sequences of hexapeptide in total. Therefore, the workload including syntheses and screening was greatly reduced.

Each sub-library was screened by HPAC. The eluted peptides with highest affinity to the FP (1–11) from each of the six sub-libraries would be analyzed by RP-HPLC and MS. The preferred sequence of each sub-library would be obtained.

	Y-R-S-K-Q-A	lead compound
Sub-library 1:	**X**-R-S-K-Q-A	preferred AA_1
Sub-library 2:	Y-**X**-S-K-Q-A	preferred AA_2
Sub-library 3:	Y-R-**X**-K-Q-A	preferred AA_3
Sub-library 4:	Y-R-S-**X**-Q-A	preferred AA_4
Sub-library 5:	Y-R-S-K-**X**-A	preferred AA_5
Sub-library 6:	Y-R-S-K-Q-**X**	preferred AA_6
preferred peptide	AA_1-AA_2-AA_3-AA_4-AA_5-AA_6	

Fig. 1. Special positional scanning peptide libraries. X represented the mixture of 18 natural L-amino acids (cysteine and tryptophan were excluded). AA_1–AA_6 represented individual preferred amino acid.

The preferred amino acids at each position of the peptide would be identified. A sequence contains the preferred amino acid residuals at each position will be generated and should have the highest affinity to the FP (1–11).

The six sub-libraries were prepared by solid-phase peptide synthesis. In order to get equimolar or close to equimolar of each peptide sequence in the libraries, the protected amino acids with a predetermined ratio were used in the peptide syntheses [21]. The peptide sequences were analyzed by MALDI-TOF-MS.

3.2. Immobilization of the FP (1–11) onto the PGMA beads

PGMA beads are cross-linked copolymer of glycidyl methacrylate and ethylene dimethacrylate. PGMA beads are monodispersed, non-porous beads with the diameter of 8 μm according to the SEM analysis and the relevant calculation. The concentration of epoxy groups on the surface of PGMA beads are 0.91 mmol g^{-1} dry beads measured by Keen's method [26]. It presents high mechanical stability, low backpressure at high flow rates, good chemical stability (pH 1–12), high hydrophilicity and low non-specific adsorption. The preparation scheme of the FP (1–11)–PGMA was shown in Fig. 2. Epichlorohydrin was used as a spacer arm in order to minimize the hindrance caused by the polymer beads which may interfere with the interactions between the FP (1–11) and peptide candidates. The quantity of epoxy groups on the surface of the epichlorohydrin-modified PGMA beads was measured using Keen's method [26] as 0.68 mmol g^{-1} dry beads. The amino groups at the N-terminus of FP (1–11) were reacted with the epoxy groups of the epichlorohydrin-modified PGMA beads in alkaline conditions. In order to increase the immobilization of FP (1–11) onto the PGMA beads, the relatively high temperature (50 °C) and long reaction time (100 h) were used. Because fusion peptide (1–11) was only a small peptide, the coupling temperature and time would not affect its property [30]. The reaction was monitored by measuring the residual peptide in the solution using the RP-HPLC method. The content of the FP (1–11) immobilized onto the PGMA reached 11.5 μmol g^{-1} dry beads.

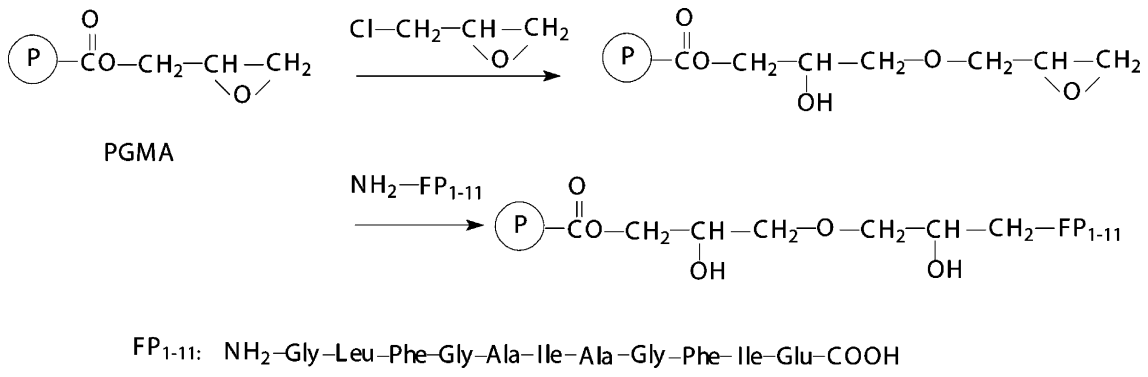

Fig. 2. Synthetic scheme of the FP (1–11)–PGMA.

Because the concentration of the FP used for the reaction with modified PGMA was equivalent to 15.5 μmol g^{-1} dry beads, the coupling efficiency of FP (1–11) onto the PGMA beads was 74.2%. Just due to the small amount of FP (1–11) was used for the coupling, the concentration of FP (1–11) on the modified PGMA was much lower than that of epoxy groups on it. Even so, the amount of FP (1–11) on the PGMA beads was enough for studying the interaction of FP with peptide libraries.

3.3. Screening of special positional scanning peptide libraries

It has been known that the low pH environment in the endosome of the host cells is needed for the conformational rearrangement of hemagglutinin to expose the fusion peptide in the fusion process of influenza virus to the host cells [9]. In order to obtain affinity candidates binding to the FP in natural conditions, pH 5.5 was chosen in the affinity chromatography screening. The special positional scanning peptide libraries were screened using the established HPAC system. The chromatographic conditions were optimized based on the interaction between YRSKQA and immobilized fusion peptide. In order to find the suitable condition for specific peptide binding to FP, the phosphate buffer of 10, 25 and 50 mmol l^{-1} (pH 5.5) were tested as the loading buffer. The samples were eluted with 50 mmol l^{-1} phosphate buffer plus 200 mmol l^{-1} of NaCl. The presence of 200 mmol l^{-1} NaCl in the elution buffer was on account of increasing the elution ability for bound peptides on the FP column so as to all the bound peptides were eluted from the affinity column after one-step elution. The affinity chromatograms of YRSKQX sub-library with three different binding buffers plus the blank run were shown in Fig. 3. With 10 mmol l^{-1} phosphate as binding buffer, a fairly sharp peak was eluted out with a retention time of 44 min, suggesting that the species in this peak has strong affinity to the FP (1–11). The majority of the peptides were found not bound to the column at all. When the buffer concentration increased to 50 mmol l^{-1}, this species observed in the 10 mmol l^{-1} phosphate buffer disappeared, which suggests that the binding is ionic strength dependent.

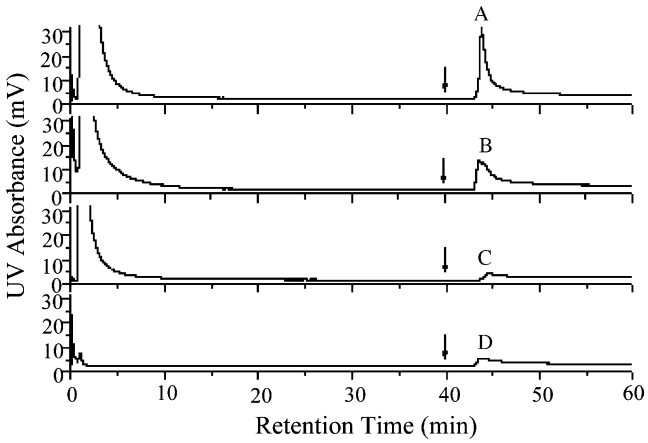

Fig. 3. Affinity chromatography of YRSKQA. Binding buffer: phosphate, pH 5.5, 10 mmol l^{-1} (A), 25 mmol l^{-1} (B), 50 mmol l^{-1} (C), and 50 mmol l^{-1} for blank run (D); elution buffer: 50 mmol l^{-1} PBS + 200 mmol l^{-1} NaCl, pH 5.5.

So, the optimized chromatographic conditions for studying YRSKQA interaction with immobilized FP (1–11) was chosen as 10 mmol l^{-1} phosphate buffer (pH 5.5). The fractions collected from the peaks of 44 min in three conditions were analyzed by RP-HPLC as shown in Fig. 4. The highest peak

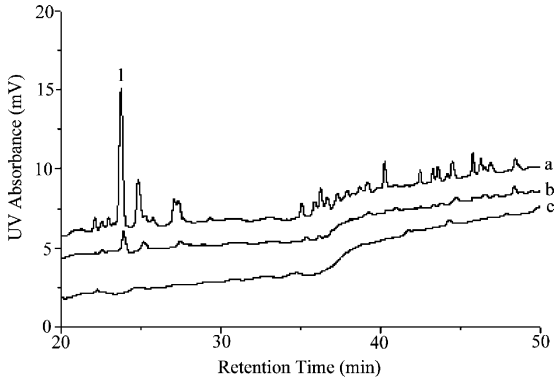

Fig. 4. RP-HPLC chromatogram of eluted fractions of YRSKQA: (a) fraction A from Fig. 3; (b) fraction B from Fig. 3; (c) fraction C from Fig. 3. Gradient: 0–30–55 min, 0–10–30% aqueous acetonitrile containing 0.1% TFA.

Table 1
Preferred amino acid residues in X position of each sub-library

Sub-library	XRSKQA	YXSKQA	YRXKQA	YRSXQA	YRSKXA	YRSKQX
Lead compound	Y	R	S	K	Q	A
Preferred amino acid	G	R	G	K	H	K
Preferred peptide	G-R-G-K-H-K					

with the biggest peak area (peak 1) in the RP-HPLC chromatogram was further analyzed using FT-ICR-MS. The *m*/*z* with double positive charges was shown as 405.235 which matched the *m*/*z* of the double charged of YRSKQK. In this library, a lysine residue in the sixth position was identified to have the highest affinity to the FP (1–11), i.e. the lysine is the preferred residue in this position.

Based on the results above, the affinity binding and elution buffers were optimized as 10 mmol l^{-1} phosphate buffer (pH 5.5) and 10 mmol l^{-1} phosphate buffer + 200 mmol l^{-1} NaCl (pH 5.5), respectively, for screening the other five sub-libraries. Using the same strategy described above, the preferred residues in position 1 to 5 were identified. The preferred residues in each position, the lead residues, and the sub-library sequences were summarized in Table 1.

The apparent dissociation constant (K_d) between GRGKHK and the FP (1–11) was 3.35×10^{-6} mol l^{-1} as measured using frontal affinity analysis [28] method in the condition of 10 mmol l^{-1} phosphate buffer. The affinity of the preferred peptide GRGKHK to the FP (1–11) is 3.3 times higher than that of the lead compound YRSKQA under the same conditions.

3.4. Design of extended peptide libraries

To test whether a higher affinity can be achieved by increasing the peptide length with preferred amino acid residues in each of the longer peptide, five extended peptide libraries (EPL) were designed and synthesized as shown in Fig. 5. The preferred peptide, GRGKHK, identified above was used as the starting compound in this process. The sequence was elongated from the N-terminus of GRGKHK. The extended peptide libraries were also synthesized by solid-phase peptide synthesis method. The EPL1 contains 18 different heptapeptide sequences, of which the first residue is one of the 18 natural amino acids (cysteine and tryptophan were excluded) and other six residues are same as the peptide GRGKHK. The library was screened using the same procedures as described above. The preferred amino acid at X position of EPL1 sequences was defined. The new peptide was used as the starting compound for library design and synthesis in the next cycle. After five cycles, a peptide with the highest affinity to the FP (1–11) was obtained with a length equal to that of the FP (1–11).

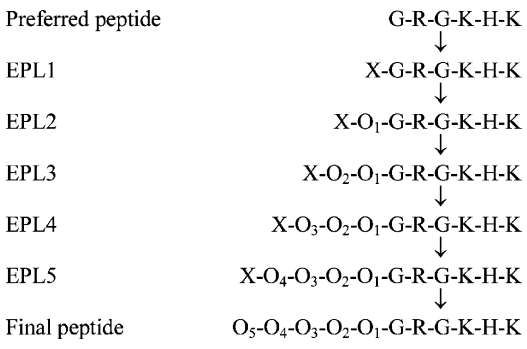

Fig. 5. Scheme of extended peptide libraries. X represented the mixture of 18 natural L-amino acids (cysteine and tryptophan were omitted). O_1–O_5 represented defined individual amino acids after screening.

3.5. Affinity interaction between extended peptide libraries and immobilized fusion peptide

In order to identify peptides with higher affinity from the extended peptide libraries, a slightly harsher condition was used. A binding buffer of 10 mmol l^{-1} phosphate (pH 5.5) plus 50 mmol l^{-1} NaCl was used in the affinity chromatography in the screening of EPL1 library. Using the same strategy described above, the preferred residue in O_1 position was identified as lysine (K) residue. In the following five cycles of screening, the NaCl concentration in the binding buffer was gradually increased. As shown in Table 2, the NaCl concentration in the binding buffer for the last cycle screening was almost the same as that in the physiological conditions.

After five cycles of screening, the peptides with the highest affinity in the EPL1–EPL5 were achieved as KGRGKHK, KKGRGKHK, RKKGRGKHK, HRKKGRGKHK and FHRKKGRGKHK, respectively. The interaction between FHRKKGRGKHK and the FP (1–11) was studied by measuring the dissociation constant of their complex using frontal affinity analysis [28]. As shown in Fig. 6, the apparent dissociation constant was calculated as 3.10×10^{-6} mol l^{-1} at the condition of 10 mmol l^{-1} phosphate buffer plus 150 mmol l^{-1} of NaCl (pH5.5). The hendecapeptide of FHRKKGRGKHK was identified to have the strongest affinity to the FP (1–11).

Table 2
Concentration of binding buffer in the screening of extended peptide libraries

	Binding buffer
EPL1	10 mM phosphate + 50 mM NaCl, pH 5.5
EPL2	10 mM phosphate + 80 mM NaCl, pH 5.5
EPL3	10 mM phosphate + 80 mM NaCl, pH 5.5
EPL4	10 mM phosphate + 100 mM NaCl, pH 5.5
EPL5	10 mM phosphate + 130 mM NaCl, pH 5.5

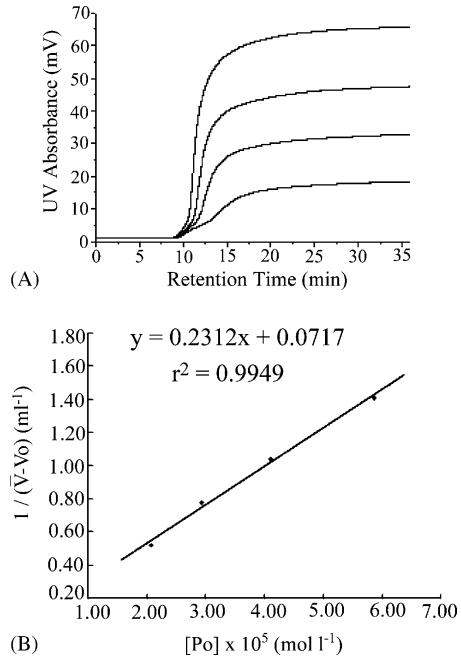

Fig. 6. (A) Frontal affinity analysis of hendecapeptide on FP (1–11) column. The concentrations of hendecapeptide were as follows: 2.07×10^{-5}, 2.93×10^{-5}, 4.10×10^{-5}, and 5.86×10^{-5} mol l^{-1}. The binding buffer was 10 mmol l^{-1} sodium phosphate (pH 5.5) containing 150 mmol l^{-1} NaCl. The flow rate was 0.5 ml min^{-1} and the detection wavelength was 280 nm. (B) Plot of $1/(\bar{V} - V_0)$ vs. $[P]_0$. The linear regression equation and correlation coefficient were shown in the figure.

3.6. Anti-influenza virus activity of screened hendecapeptide

To verify the ability of hendecapeptide to neutralize viral infectivity, the MDCK cells were inoculated with three different H1N1 strains of influenza A virus (A/HUFANG/7/1999, A/CNIC/143/2001 and A/GUIZHOU/259/2000) in the presence of hendecapeptide at 35 °C for 48 h, respectively. The supernatants were collected from the cells and assayed by erythrocyte agglutination. Neutralization of virus by the screened peptide was calculated by reduced number of viral-induced CPE and erythrocyte agglutination relative to the controls inoculated in the absence of the peptide at the same condition. It was demonstrated that the virus replication was inhibited after the virus was treated with the screened peptide. FHRKKGRGKHK showed 1 log unit inhibitory activity against influenza A virus. The data confirm that the hendecapeptide FHRKKGRGKHK has a definite inhibition on influenza A virus replication.

4. Conclusion

The novel method of using affinity chromatography and peptide libraries in screening inhibitors for influenza A virus has been proved feasible. The strategies of using the antisense peptide based special positional scanning peptide libraries and the following extended peptide libraries were confirmed as very valid approaches in affinity chromatographic screening. Using this approach, much smaller libraries are needed to identify target compounds. The hendecapeptide revealed the highest binding affinity to the FP (1–11) of influenza A virus in the physiological buffer conditions and also expressed defined anti-influenza virus activity. The authors believe that the strategy used in this report can also be applied in searching inhibitors of other virus such as HIV, Ebola, etc. which use the same mechanism in the fusion process during the infection.

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