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Study on the degeneracy of antisense peptides using affinity chromatography

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Abstract

The degeneracy of antisense peptides was studied by high-performance affinity chromatography. A model sense peptide (AAAA) and its antisense peptides (CGGG, GGGG, RGGG, SGGG) were designed and synthesized according to the degeneracy of genetic codes. An affinity column with AAAA as the ligand was prepared. The affinity chromatographic behaviors of antisense peptides on the column were evaluated. The results indicated that model antisense peptides have clear retention on the immobilized AAAA affinity column. RGGG showed the strongest affinity interaction. Similar result was obtained from another experiment that Arg-substituted antisense peptide of fusion peptide (1–11) of influenza virus A was also shown the highest affinity binding to immobilized fusion peptide.

Keywords: Affinity chromatography; Immobilized peptides; Degeneracy; Antisense peptides; Peptides; Amino acids

1. Introduction

Antisense peptides are defined as the sequences of amino acids encoded in the antisense strand of DNA (Fig. 1). In general, DNA has a coding strand and a non-coding strand. The coding strand is called sense-DNA chain and generates a peptide or a protein, which is called sense peptide or sense protein. The non-coding strand is called antisense DNA chain and usually cannot generate a peptide or a protein in almost all organisms, except in some simple organisms such as phage and viruses [1]. But antisense peptides can be synthesized chemically. There were two main approaches to study the mechanism of interaction between sense peptide and its antisense peptide. One is the Mekler–Biro–Blalock (MBB) approach [2–4], the other is the Root-Bernstein (R-B) approach [5,6]. Mechanistic understanding of antisense peptide recognition remains incomplete, but the antisense peptides generated from readings of

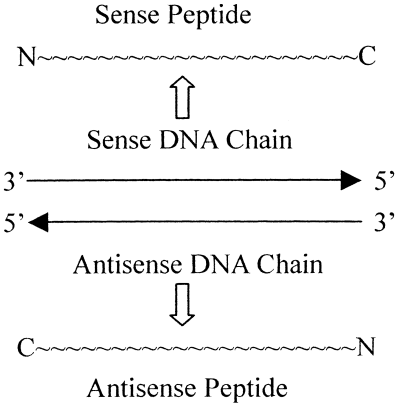

Fig. 1. Relationship between sense and antisense peptides encoded by sense and antisense DNA chains.

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the non-coding strand of DNA have displayed biological activities [7].

Affinity chromatography is considered a specific mode of HPLC since it is based on molecular recognition. Affinity ligands can be classified into two groups. The first group contains group-specific ligands, such as amino acids, dyes, lectins, nucleotides and metal ions. The second group contains specific ligands, including antibody for its antigen, inhibitor for its enzyme, hormone for its receptor, etc. There are some disadvantages to the use of the above-mentioned ligands; for instance, low specificity of group-specific ligands, high cost, short lifetime, further contamination of the final products when monoclonal antibodies are used as the ligands [8].

Antisense peptides have been found experimentally to bind to sense peptides and proteins with significant selectivity and affinity. This kind of recognition has been observed and applied in many systems, especially in the separation of peptides and proteins using affinity chromatography [1,9]. Synthetic antisense peptides encoded in the antisense strands of DNA corresponding to 1–14, 42–54 and 103–115 fragments of human interferon-β sequence were applied in the purification of recombinant human interferon-β from a mammalian cell culture, permitting a 10-fold purification of the protein [10]. Antisense peptide encoded in the antisense strand of DNA corresponding to the N-terminal 20 residues of pro AVP/BNPII was immobilized and used to separate Arg^8-vasopressin and oxytocin, neurophysin II and I in bovine posterior pituitary acid extract [11]. Although there were several successful experiments on the use of antisense peptide-based affinity separations, it is still difficult to directly obtain an antisense peptide with high binding constant from its sequence of corresponding sense peptide. Some efforts to increase the affinity interactions between sense peptide and antisense peptide have been carried out [12–14]. We noticed that there is a degeneracy of genetic codes, therefore a degeneracy of antisense peptides might exist. We hope to demonstrate the existence of antisense peptides' degeneracy and to increase the affinity recognition between sense peptide and its antisense peptides by systematically modifying the sequence of antisense peptides according to the degeneracy of genetic codes in order to develop a simple and effective method for selecting affinity ligands with low cost, long life-time and high bio-specificity.

In this paper, a sense peptide and its four antisense peptides were designed as models of affinity interaction. The sequence of antisense peptides was varied based on the degeneracy of genetic codes. The affinity interaction between model sense and the corresponding antisense peptides was evaluated by high-performance affinity chromatography. Fusion peptide (1–11, FP11) from influenza virus A and its antisense peptides were also used in this study.

2. Experimental

2.1. Chemicals and samples

9-Fluorenylmethoxycarbonyl (FMOC)-derivatized amino acids, 4-hydroxymethylphenoxyacetic (HMP) resin (Wang resin), *N*,*N*′-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), *N*-hydroxybenzotriazole (HOBt) were purchased from Siam (USA). Trifluoroacetic acid (TFA) and ethanedithiol were obtained from Sigma (USA). Acetonitrile (HPLC grade) was from Fisher (USA). Piperidine, dichloromethane (DCM), *N*,*N*′-dimethyl formamide (DMF) and other chemicals were of analytical grade and purchased from Beijing Chemical Factory (China). Monosized non-porous poly (glycidyl methacrylate) beads (PGMA) containing active epoxide groups were made in our laboratory.

2.2. Solid-phase peptide synthesis

The FMOC strategy [15] of solid-phase peptide synthesis was used for peptide syntheses. The syntheses were carried out manually. Wang resin was used as the support. The attachment of the first amino acid, glycine, alanine, lysine or glutamic acid, to Wang resin was accomplished using symmetrical anhydride and 4-dimethylaminopyridine as a catalyst. The subsequent coupling cycles were performed in the presence of dicyclohexylcarbodiimide and hydroxybenzotriazole. After completion of synthesis cycles, the peptide resins were dried under high vacuum for 4 h. The peptides were cleaved from the resins using 10 ml of TFA–water (95:5) or TFA–

ethanedithiol–water (95:2.5:2.5) and left to stand at room temperature with occasional swirling from 1.5 to 3 h according to the peptide sequences. The mixture was then filtered and evaporated to 1 ml. The peptides were precipitated by adding cold diethyl ether. The collected precipitates were dissolved in acetonitrile–water solution and lyophilized.

2.3. Purification and characterization of peptides

The synthesized crude peptides were purified and identified by RP-HPLC and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS. The purified peptides were also analyzed on a Phenomenex C_{18} column (250×4.6 mm I.D.) at a flow-rate of 1.0 ml/min using a isocratic elution of 5% acetonitrile in 0.1% aqueous TFA from zero time to 10 min and a gradient from 5% acetonitrile in 0.1% aqueous TFA at 10.01 min to 60% acetonitrile in 0.1% aqueous TFA at 40 min. The effluent was monitored at 220 nm (0.05 AUFS)

2.4. Preparation of high-performance affinity columns

2.4.1. Preparation of affinity packing with AAAA as the ligand

Poly(glycidyl methacrylate) beads (PGMA) [16] (7 μm, monosized, non-porous) containing active epoxide groups were used as the affinity support. PGMA resin (2 g) was reacted with $NaIO_4$ in acetic acid solution (10%) for 4 h, and then reacted with excess ethylenediamine in the presence of $NaBH_3CN$ for 3 h to get a spacer arm with a free amino group. $NaBH_4$ was added to reduce the unreacted aldehyde groups. The affinity ligand AAAA was elongated from the NH_2 groups of PGMA resin using FMOC solid-phase peptide synthesis method. Kaiser reagent was used to detect the reaction completion. The packing used for blank column was prepared as described above, except peptide elongation.

2.4.2. Preparation of affinity packing with FP11 as the ligand

Sixteen mg of FP11 was dissolved in 16 ml of 0.1 *M* $NaHCO_3$–Na_2CO_3 buffer at pH 9.5, and then 1.65 g of PGMA resin were added to the solution. The suspension was incubated for 50 h at 37°C. In a similar way, the packing for blank column was prepared by reacting 1.65 g of PGMA resin with 16 ml of 0.1 *M* $NaHCO_3$–Na_2CO_3 buffer at pH 9.5.

The packings, immobilized AAAA and FP11 on PGMA resin, were packed into a 70×4-mm I.D. stainless steel column at a pressure of 200 kg/cm^2, respectively. The blank columns were also packed using the same method.

2.5. Affinity chromatographic conditions

The HPLC system consisted of a Shimadzu LC-$10AT_{VP}$ pump, a Shimadzu DGU-14A degasser, a Shimadzu SPD-10A UV–Vis detector, a Rheodyne 7725I sample injection valve and a WDL-95 chromatographic work station. One mg/ml of peptide solution was prepared with 3 times distilled water as the samples. The eluents were 5 and 10 m*M* phosphate buffers with pH 2.0, 5.5, 6.0, 7.0, 10.0, respectively. Flow rate was 0.1 ml/min in the isocratic elution mode. The antisense peptides were detected at 220 nm.

3. Results and discussion

3.1. Design of the model sense peptide and its antisense peptides

Based on the degeneracy of genetic codes, one amino acid can be encoded by more than one codon in most cases. It means an amino acid in a sense peptide chain may have a group of complementary amino acids. More than one antisense peptide could be chemically synthesized in this case. We call this the degeneracy of antisense peptides.

In order to study the interaction of a sense peptide and its antisense peptides as well as the degeneracy of antisense peptides, one model sense peptide and its model antisense peptides were designed based on the degeneracy of genetic codes and the principle of complementary base pairs. Alanine was chosen as the model sense amino acid, first because there are no basic and acidic side chains in its structure, moreover it has relatively many antisense amino acids. The direct-readout antisense amino acids corresponding to the complementary codon from 5′ to 3′ of sense Ala were Arg, Cys, Gly and Ser. The

Table 1
Model sense and antisense amino acids with their codons

Sense amino acid	Codon 5′–3′	Complementary codon		Antisense amino acid	
		5′–3′	3′–5′	5′–3′	3′–5′
Ala	GCG	CGC	CGC	Arg	Arg
	GCA	UGC	CGU	Cys	Arg
	GCC	GGC	CGG	Gly	Arg
	GCU	AGC	CGA	Ser	Arg

antisense amino acid from the complementary codon 3′ to 5′ was only Arg (Table 1). We noticed that some small peptides showed high biological activities in nature, such as TRH (tripeptide) [17], tuftsin (tetrapeptide) [18], so oligotetrapeptides were chosen as the model sense peptide. Sequence variants of antisense peptides were first made at the N-terminal of every antisense peptide in order to simplify the structure modification of antisense peptides, as well as to observe the affinity difference caused by one amino acid variance. The tetrapeptide AAAA was designed and synthesized as the sense peptide and used as the affinity ligand. Four tetrapeptides containing Arg, Cys, Gly and Ser at the N-terminal were designed and synthesized as the antisense peptides according to the degeneracy of Ala. Their sequences are shown in Table 2. An irrelative peptide with non-polar amino acids, VFLAG, was also designed and synthesized because the antisense amino acids of alanine are most polar amino acids with or without charge.

3.2. Preparation of affinity medium with sense peptide (AAAA) as the ligand

PGMA beads containing active epoxide groups were used as the affinity support. PGMA resin is produced as monosized, non-porous beads and possesses high mechanical stability, low back pressure at high flow-rates good chemical stability (pH 1–12), hydrophilicity and low non-specific adsorption after the epoxide groups are hydrolyzed to diol groups. Modification of the resin is quite easy due to the presence of active epoxide groups on the surface of PGMA beads. PGMA beads are very suitable for high-performance affinity chromatography. Ethylenediamine was chosen as a spacer arm. The ligand AAAA was synthesized directly on the modified, free amino group containing PGMA resin by FMOC solid-phase synthesis (Fig. 2). The solid-phase techniques have been greatly refined over the years to allow the creation of essentially any peptide in a consistently high yield. The test by Kaiser reagent indicated that the synthesis reaction of ligand AAAA is complete. Further evidence for the successful synthesis of the medium was given by X-ray photoelectron spectrometric analysis.

Table 2
Sequences of model sense peptide and model antisense peptides

Sense peptide	Antisense peptide (AS)
Ala–Ala–Ala–Ala (AAAA)	**Arg–Gly–Gly–Gly (RGGG)**
	Cys–Gly–Gly–Gly (**CGGG**)
	Gly–Gly–Gly–Gly (**GGGG**)
	Ser–Gly–Gly–Gly (**SGGG**)

3.3. Affinity interaction of immobilized AAAA with its antisense peptides

Affinity chromatographic study of the recognition between the sense peptide and its antisense peptides has made it possible to define quantitative properties of these interactions [12] and identify the weak interactions between sense peptide and its antisense peptides. The affinity interactions between immobilized sense peptide AAAA and its four antisense peptides were evaluated by high-performance affinity chromatography with 5 m*M* phosphate buffer at pH 2.0, 6.0 and 10.0 as the eluents. The data are shown

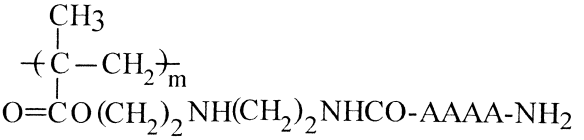

Fig. 2. Model of AAAA immobilized PGMA resin.

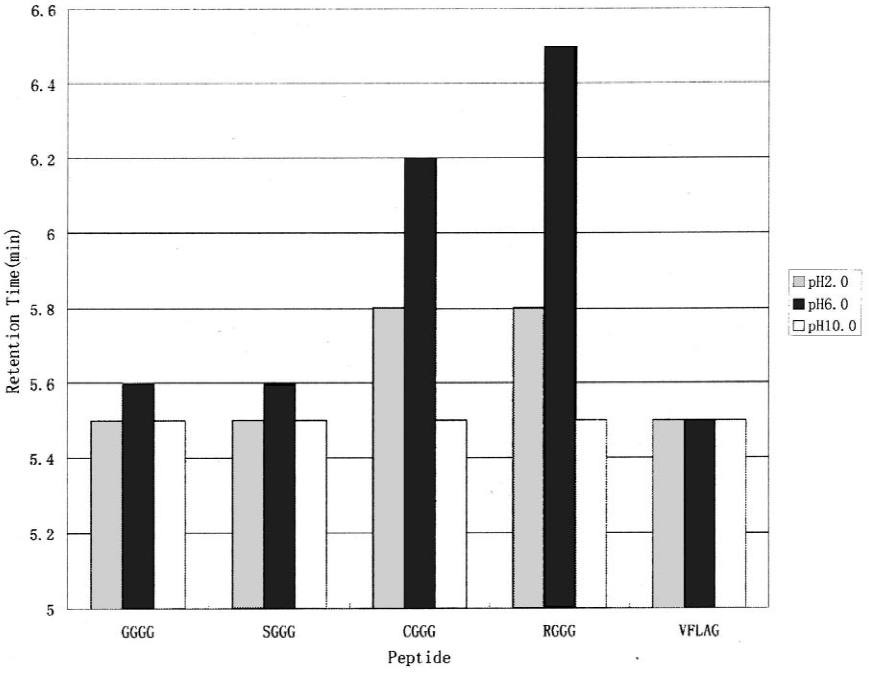

Fig. 3. Affinity interaction of antisense peptides and irrelative peptide with immobilized sense peptide AAAA. Chromatographic condition as described in Section 2.5.

in Fig. 3. All four antisense peptides have measurable affinity for the immobilized sense peptide at pH 6.0 and do not bind to sense peptide AAAA at pH 10.0. Only RGGG and CGGG expressed weaker affinity at pH 2.0 than at pH 6.0. The antisense peptide containing Arg residue showed the strongest binding to its sense peptide and the most notable change at different pH values. The order of retention for antisense peptides on the column packed with AAAA immobilized PGMA was RGGG>CGGG> GGGG, SGGG. There was no retention for the irrelative peptide VFLAG at any of the three pH values. Reference experiments using the blank column were carried out at the same conditions. No retention was observed on the blank column.

Four antisense peptides and the irrelative peptide VFLAG were analyzed by reversed-phase HPLC. The retention time is shown in Table 3. The hydrophobic difference of antisense peptides is only caused by the N-terminal amino acid residues, Arg, Cys, Gly and Ser.

Irrelative peptide VFLAG is the most hydrophobic one according to retention time (longest) on the reversed-phase column, and lack of retention on the AAAA immobilized column. RGGG and CGGG have the same retention time on a RP column, but RGGG showed higher binding affinity to immobilized AAAA than CGGG. Therefore, the retention of antisense peptides on the affinity column is not

Table 3
Retention times of peptides in RP-HPLC analysis

Peptide designation	No. of residues	Retention time on RP-HPLC (min)
SGGG	4	3.6
GGGG	4	3.7
CGGG	4	4.1
RGGG	4	4.1
AAAA	4	5.3
YSSKQA	6	9.8
YGSKQA	6	10.0
YCSKQA	6	11.8
YRSKQA	6	9.0
FP11	11	37.9
VFLAG	5	28.8
NYQEAK	6	13.9

Chromatographic conditions are described in Section 2.3.

complete under hydrophobic mode. The affinity medium possesses amino groups from immobilized AAAA chains and showed anion-exchange behavior at pH 2.0 and 6.0. RGGG has the strongest retention on the immobilized AAAA column although it has a positive charge at pH 6. This means that binding affinity is independent of the ion-exchange mode. None of the samples can be eluted from the affinity column according to the size-exclusion mode because non-porous PGMA beads were used as support.

3.4. Design of sense peptide, fusion peptide 1–11 and its antisense peptides

Another example was chosen to confirm the affinity between the sense peptide containing Ala and the antisense peptides containing Arg, Cys, Gly and Ser. Fusion peptide (FP11) of hemagglutinin of influenza virus A [Hong Kong/156/97(H5N1)] was used as the sense peptide. Four antisense hexapeptides were designed according to the complementary mRNA of sense peptide and modified at position one by Arg, Cys, Gly and Ser residues, respectively (Fig. 4).

Fig. 4 denotes the relationship of antisense peptides to sense peptide. CUA encoded Leu, but UAG, the complementary codon of CUA, is a termination codon and does not encode any amino acid. Leu was also encoded by UUA, UUG, CUU, CUC and CUG according to the degeneracy of genetic codes, so the antisense amino acids of Leu are Gln, Lys and Glu. Gln was used in this study because there is no charge in its structure. The read-out antisense amino acid of Gly at C-terminal was Ser. Ala was also the antisense amino acid of Gly according to the degeneracy

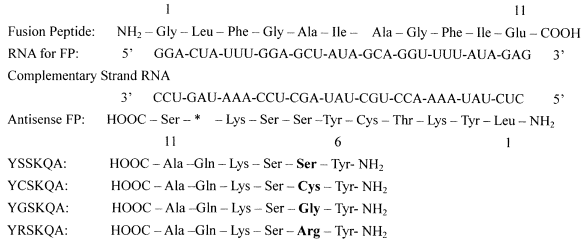

Fig. 4. Amino acid sequence of antisense peptides derivatized from the corresponding RNA of fusion peptide. *UAG is the termination codon.

of genetic codes. Ser was replaced by Ala, because that simplified peptide synthesis. In this experiment, we wanted to emphasize the affinity interaction between sense peptide containing Ala and antisense peptides containing Arg, Cys, Gly, Ser. Therefore, we made antisense peptides with respect to fusion peptide 1–6 containing only one Ala residue at position five. The read-out antisense amino acid of Ala was Ser. Ser was replaced by Cys, Gly and Arg to form four antisense peptides (6–11), which correspond to fusion peptide 1–6. Antisense peptides with 11 amino acid residues were also studied in other experiments (data not shown). They were different from the tetrapeptide experiment in that Arg, Cys, Gly, Ser were in the middle of antisense peptide chains with respect to the position of Ala in the sense peptide chain. NYQEAK, which contains every polar amino acid was designed as the irrelative peptide. FP11, four antisense hexapeptides (YGSKQA, YCSKQA YSSKQA YRSKQA) and the irrelative peptide were synthesized by the solid-phase method and purified to chromatographic homogeneity by RP-HPLC. These peptides were identified by MALDI-TOF-MS. The hydrophobicity of hexapeptides was measured by RP-HPLC analysis. The data are shown in Table 3.

3.5. Recognition properties of immobilized FP11

The fusion peptide (1–11) was used as the sense peptide and immobilized to the PGMA resin directly. The irrelative peptides, VFLAG, NYQEAK were used in the study. The affinity interaction was investigated in the presence of 10 m*M* phosphate buffer at pH 2.0, 5.5, 7.0, 10.0, respectively (Fig. 5). There was high affinity at neutral pH (pH 5.5, 7.0). The native antisense peptide (YSSKQA) did not show the highest interaction with its sense peptide FP11. The binding ability of antisense peptides to immobilized sense peptide FP11 were YRSKQA> YCSKQA>YGSKQA, YSSKQA. The antisense peptide with Arg residue showed the highest recognition with immobilized FP11 at pH 7.0. Parallel experiments were carried out using the blank column. No retention was observed in the blank experiment.

Compared with the affinity model of RGGG, CGGG, GGGG, SGGG with AAAA, the binding orders of two models were found to be the same,

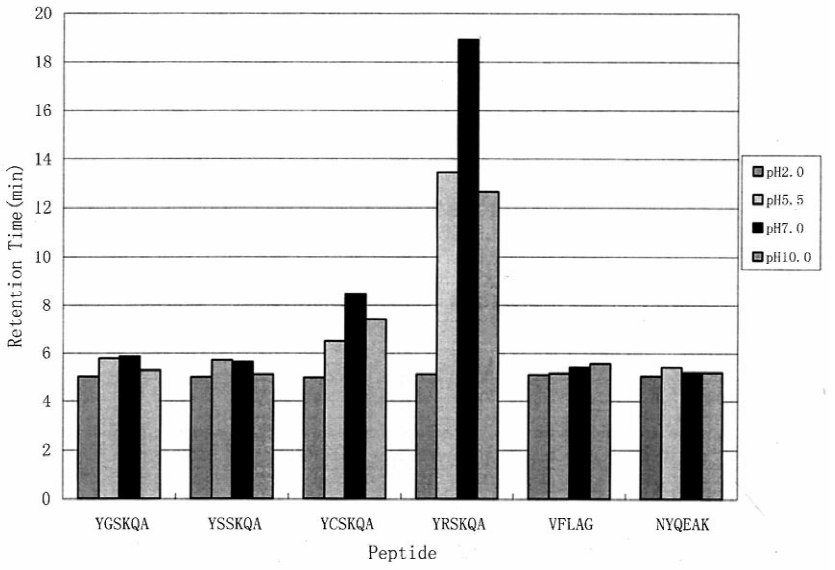

Fig. 5. Affinity chromatographic behavior of four antisense peptides and two irrelative peptides on FP11 affinity column. Chromatographic condition as described in Section 2.5.

RGGG>CGGG>GGGG, SGGG; YRSKQA> YCSKQA>YGSKQA, YSSKQA. Antisense peptides containing Arg residue have the strongest affinity whether Arg is at the N-terminal or in the middle of peptide sequences, whether the affinity columns show anion- or cation-exchange properties. There is no direct relation between the hydrophobic property and the affinity binding of antisense peptides. The selectivity was confirmed by the lack of retention of several irrelative peptides on the immobilized AAAA and FP11 columns. There is no evidence that every sense peptide has detectable affinity interactions with its antisense peptide [8]. In this study, antisense peptides containing Gly and Ser residues did not show remarkable binding with their sense counterparts, but antisense peptides containing Arg and Cys residues show significant binding to their sense peptides. The purpose of studying the degeneracy of antisense peptides is to demonstrate if the modified antisense peptides keep, decrease or increase the affinity binding to their sense peptide. It is possible that affinity and selectivity between antisense peptides and sense peptide would be increased by modifying the amino acid sequence of antisense peptide according to the degeneracy of antisense peptide.

Studies of degeneracy of model sense peptides and the corresponding antisense peptides containing other kinds of amino acids are being carried out in our laboratory. If all kinds of peptides show degeneracy, it would be simple and effective to design and select antisense peptides as ligands to improve the affinity recognition of native peptides or proteins in affinity chromatography by varying the amino acids in antisense peptide chains based on the degeneracy, especially when the direct readout antisense peptide did not show the high affinity binding.

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