

Reducing Allergenicity to Arginine Kinase from Mud Crab Using Site-Directed Mutagenesis and Peptide Aptamers

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Supporting Information

ABSTRACT: The mud crab (*Scylla paramamosain*) is widely consumed but can cause a severe food allergic reaction. To reduce allergenicity to arginine kinase (AK), site-directed mutagenesis was used to destroy disulfide bonds or mutate critical amino acids of conformational epitopes. Three hypoallergenic mutant AKs (mAK1, mAK2, and mAK3) were generated, with the immunoreactivity decreasing by 54.2, 40.1, and 71.4%, respectively. In comparison to recombinant AK (rAK), the structure of mAKs was clearly changed. Additionally, antisense peptides were designed on the basis of linear epitopes and pepsin-cutting sites of AK. Five peptide aptamers were screened by molecular docking and then analyzed by the immunoglobulin E inhibition enzyme-linked immunosorbent assay and human Laboratory of Allergic Diseases 2 mast cell degranulation assay. The peptide aptamers could significantly inhibit allergenicity of rAK and mAKs, and the inhibitory effect of peptide aptamer 3 was slightly better than the others. These results provide synergistic methods to reduce allergenicity to AK, which could be applied to other shellfish allergens.

KEYWORDS: arginine kinase, peptide aptamers, reducing allergenicity, *Scylla paramamosain*, site-directed mutagenesis

INTRODUCTION

In recent decades, the prevalence of food allergies has increased worldwide.¹ Shellfish is one of the eight major sources of food allergens that can cause an immunoglobulin E (IgE)-mediate type I hypersensitivity reaction.^{2,3} Different from allergies caused by eggs and milk, shellfish allergy is a long-lasting disorder that can significantly impact the quality of life of the patient.^{4,5}

The mud crab, *Scylla paramamosain*, is widely consumed in China, especially in the coastal areas. Arginine kinase (AK) in shellfish muscle has been identified as a major allergen that can cause cross-reactivity between species. Yu et al. identified that AK is an important allergen from *Penaeus monodon* by molecular cloning and immunoblotting.⁶ Mao et al. also identified AK as a major allergen in *S. paramamosain* that triggered strong IgE reactivity.⁷ Moreover, there are six cysteine residues in AK, and Cys₂₀₁ and Cys₂₇₁ form an intramolecular disulfide bond, which is essential for maintaining the structural stability of AK.⁸ As reported, Gattis et al. mutated the active site at C₂₇₁ in AK from horseshoe crabs, which changed its catalytic activity.⁹ However, there are few studies altering the allergenicity of AK by mutating these cysteine residues.

The antigenicity of an allergen depends upon its epitopes.¹⁰ An epitope is the region of an allergen that is recognized by a specific antibody and is usually on the surface of the antigen.¹¹ On the basis of the composition of amino acids as to whether it is contiguous or not, an epitope is categorized as linear or

conformational.¹² Currently, the most common technique to modify allergens is site-directed mutagenesis. For example, a hypoallergenic variant of Rho 1 was generated by mutating residues of the IgE epitope that were crucial for antibody binding.¹³ A hypoallergenic form of shrimp tropomyosin (TM) was constructed in which 49 point mutations were introduced by site-directed mutagenesis.¹⁴ The conformational and linear epitopes of AK from *S. paramamosain* are well-documented in our previous research,¹⁵ while how to generate a hypoallergenic variant based on conformational epitopes of AK using site-directed mutagenesis requires further study.

An aptamer is a small sequence of oligonucleotides or amino acids that is capable of binding target molecules.¹⁶ In the past, aptamers were widely used as therapeutic and diagnostic tools as a result of their high specificity and affinity.¹⁶ Currently, aptamers are also applied to study allergens, such as in their detection. Low et al. produced a DNA aptamer that could bind with the IgE epitope of Asp f 1 from *Aspergillus fumigatus*, and the allergen measurement methods could be developed to indicate the allergenicity of allergens.¹⁷ A DNA aptamer was used in biosensor assays to detect tropomyosin from *Penaeus vannamei* by Zhang et al.¹⁸ However, the use of aptamers to

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reduce the allergenicity of allergens has not been studied before to our knowledge.

In addition, for aptamer screening, molecular docking techniques have been extensively applied. Kadioglu et al. identified a peptide aptamer by molecular docking that could target translational controlled tumor proteins in leukemia cells.¹⁹ Antisense peptide technology is a valuable tool for deriving new biologically active molecules and performing peptide–receptor modulation.²⁰ By definition, a sense peptide is coded by the nucleotide sequence of the sense strand of DNA, while an antisense peptide is coded by the nucleotide sequence of the complementary (antisense) strand.²¹ Many studies have identified specific interactions between a sense peptide and its antisense peptide.^{22,23} An antisense peptide was designed that could target the receptor-binding site of human erythropoietin by Stambuk et al.²⁰ Because that antisense peptide had high specificity and efficacy with its sense peptide, it has the potential to act as an aptamer that can specifically bind to epitopes of AK.

In this study, we aimed to reduce the allergenicity of AK from *S. paramamosain* by changing its structure and modifying its antigen epitopes. The disulfide bond of AK was destroyed by site-directed mutagenesis. For conformational epitopes, site-directed mutagenesis was used to generate hypoallergenic variants. In addition, antisense peptides were designed on the basis of the linear epitopes and pepsin cutting sites of AK and were analyzed by molecular docking, to screen for peptide aptamers that specifically bind to linear epitopes of AK. Moreover, the inhibitory effects of peptide aptamers on the immunobinding reactivity of wild and mutant AK were detected by the inhibition enzyme-linked immunosorbent assay (ELISA) and human Laboratory of Allergic Diseases 2 (LAD2) mast cell degranulation assay.

MATERIALS AND METHODS

Materials. Mud crabs were purchased at Jimei Market (Xiamen, Fujian, China). The Eastep Super Total RNA Extraction Kit, Mutantbest Kit, and DNA Purification Kit were purchased from TaKaRa (Dalian, Liaoning, China). The peptides were synthesized by Cell-mano Biotech (Hefei, Anhui, China). Horseradish-peroxidase-labeled goat anti-human IgE antibody and goat anti-rabbit immunoglobulin G (IgG) antibody were purchased from Southern Biotech (Birmingham, AL, U.S.A.). Enhanced chemiluminescence (ECL) substrate was purchased from Pierce (Rockford, IL, U.S.A.).

Patient Sera. Sera from 13 crab-allergic patients were provided by Xiamen Medical College Affiliated Second Hospital (human ethical approval XSH2012-EAN019, Xiamen, China) and Xiamen Maternity and Child Health Care Hospital (human ethical approval KY-2018-018 and KY-2019-014). Their specific IgE antibodies to crab (Table 1) were quantified *in vitro* using an ImmunoCAP (Phadia AB, Uppsala, Sweden). Serum with a specific IgE of >0.35 (kU/L) was defined as positive. Adult patients and the parents of minors signed informed consent. All sera were stored at −20 °C until analysis.

Site-Directed Mutagenesis of AK from *S. paramamosain*. Specific primers (Table 2) were designed and synthesized according to the sequence of AK from *S. paramamosain* (GenBank accession number JQ031765.1). Total RNA was extracted from muscle and then reversed transcribed to cDNA, which served as a template for polymerase chain reaction (PCR). The Mutantbest Kit was used to carry out site-directed mutagenesis for generating mutants. The universal DNA Purification Kit was applied to purify the PCR products. Mutant genes were subcloned into pET-28a and verified by sequencing. The clones were transformed into *Escherichia coli* BL21 (DE3) cells, and the expression strain was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). Expression products were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–

Table 1. Specific IgE Levels and Symptoms of the Crab Allergic Patients and a Non-allergic Individual

serum ID	age (years)	sex ^a	specific IgE (kU/L) ^b	symptoms
814	17	F	1.6	pruritus and nausea
804	18	F	61.7	nausea, dyspnea, and diarrhea
996	17	F	2.8	asthma and dyspnea
838	18	F	45.1	urticaria, nausea, emesis, and diarrhea
186	19	F	11.1	nausea and emesis
646	35	M	32.5	dermatitis
172	27	F	0.6	eczema
211	18	F	751.1	c
212	37	F	107.1	c
267	27	F	201.2	anaphylactic rhinitis
281	31	F	119.0	c
303	34	F	310.8	c
168 ^d	24	M	0.06	c

^aM, male; F, female. ^bA serum with a specific IgE of >0.35 (kU/L) is defined as positive. ^cNo symptom. ^dThe person was a non-allergic individual.

Table 2. Oligonucleotide Primers Used in PCR^a

primer	oligonucleotide sequence (5′ → 3′)
F	CATATGATGGCTGACGCTGCTACCAT
R	GCTGACTAGTTCTAAGCTCTTCTTACGTC
F ₁ -33K	AAGTACCTCACC <u>GCG</u> TCTGTGTTCG
R ₁ -33K	CAGGAGAGACTTGCAGTCAGTGGCA
F ₂ -174T	TCACTGGCATG <u>GCG</u> AAGGATGTGC
R ₂ -174T	GATAGTATGTGCCCTTGAGCTCACC
F ₃ -201C	CGCCAATGCC <u>GCG</u> CGCTACGCGCCCA
R ₃ -201C	GCGGCCTGCAGGAAGCG

^aThe nucleotide changes are underlined. CATATG and GCTGAC are restriction sites for *Nde* I and *Sal* I enzymes, respectively.

PAGE) and western blot using rabbit anti-*S. paramamosain* AK polyclonal antibody. Denatured protein was dissolved in 10 mM Tris (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 10 mM NaCl, and 8 M urea, which involved sequential dialysis until no urea remained. Mutant AK (mAK) and recombinant AK (rAK) were purified using nickel–nitrilotriacetic acid (Ni–NTA) resin according to the method of Mao et al.⁷

Disulfide Bonds and IgE Immunoreactivity Analysis.

Disulfide bonds in mAKs were detected according to the method published by Yano et al.²⁴ rAK and mAKs were incubated with iodoacetamide to a final concentration of 5 mM for 1 h at room temperature. Then, 0.3 μL of 150 mM DTT was added to the protein, and the mixture was incubated for 1 h at room temperature. A total of 2 μL of 150 mM mBBR fluorescent reagent was added. The mixture was allowed to stand for 15 min at room temperature and was then ready for separation by SDS–PAGE. The gel was destained by 30% methanol and 5% acetic acid until protein bands were visible.

The specific IgE-binding activity of rAK and mAKs was detected by dot blots using crab-allergic patient sera as the primary antibody, referencing the method of Liu et al. A total of 2 μL of 1 mg/mL of purified rAK or mAKs was spotted on nitrocellulose membranes and blocked with 5% skim milk for 1.5 h.²⁵ Then, crab-allergic patient serum (1:5 dilution) was spotted on the membranes for 1.5 h at 37 °C. The secondary antibody horseradish-peroxidase-labeled goat anti-human IgE antibody (1:2000 dilution) was incubated with the nitrocellulose membranes for 1 h at 37 °C. The immunoassay was developed with ECL substrate, which contained 0.01 M luminol and 0.1% H₂O₂ mixed with the same volume. The nitrocellulose membranes were detected by the chemiluminescence fluorescence imaging system (Protein Simple, Silicon Valley, CA, U.S.A.) after

incubated with the substrate for 2 min. The results of the dot blot were quantized and shown as a histogram, referencing the method of Wai et al.¹⁴ Briefly, on the basis of the intensity of dots shown on nitrocellulose membranes, the strong, middle, and weak intensities of dots were evaluated as 3, 2, and 1 scores, respectively. The scores evaluated by three individuals were performed for statistical analysis.

The inhibition ELISA was performed to further analyze the IgE-binding activity of mAKs using the serum pool (sera from crab-allergic patients in Table 1) according to the method of Hu et al.²⁶ rAK (200 ng/well) was coated on the 96-well ELISA plate and incubated at 4 °C overnight. Inhibitors (rAK or mAKs) were prepared the same as rAK (200 ng/well) and preincubated with the same volume of sera (1:5 dilution) for 2 h at 37 °C. After that, the serum pool with inhibitors were added in the plate incubated for 2 h at 37 °C. In inhibition ELISA, the positive control was rAK without inhibitors and the negative control was coated with coating buffer. The inhibition rates were calculated as

$$1 - (\text{experimental group} - \text{negative control}) / (\text{positive control} - \text{negative control})$$

Circular Dichroism (CD) Spectra and Surface Hydrophobicity Analysis. The secondary structures of rAK and mAKs were detected with a CD spectrophotometer (Applied Photophysics, Ltd., Surrey, U.K.) at 25 °C. The proteins were concentrated to 0.5 mg/mL in 20 mM phosphate-buffered saline (PBS) at pH 7.4. The effects of the temperature on rAK and mAK secondary structures were detected by CD spectra, ranging from 20 to 100 °C. The CD spectra were collected from 185 to 255 nm at a scanning rate of 100 nm/min with a bandwidth of 1.0 nm at a heating rate of 1 °C/min.

The surface hydrophobicity of rAK and mAKs was measured using a hydrophobic fluorescence probe, 8-anilino-1-naphthalenesulfonate (ANS), and the measurement was performed according to the method by Tong et al.²⁷ A total of 1.0 mL of 0.1 mg/mL rAK and mAKs was incubated with 5 μ L of 5 mM ANS solution for 1 h at room temperature. Benchmark 96 (Bio-Rad, Hercules, CA, U.S.A.) was used to detect the relative fluorescence of rAK and mAKs. The excitation wavelength was 390 nm, and the emission wavelength was from 400 to 600 nm with a scanning speed of 25 nm/s and a bandwidth of 5.0 nm.

Pepsin Cutting Sites and Antisense Peptide Analysis. The program ExPASy peptide cutter, available at http://web.expasy.org/peptide_cutter, was used to analyze the pepsin-cutting sites of AK. The peptide cutter predicted potential cleavage sites of pepsin activity to analyze whether they affected the integrity of linear epitopes of AK.

Specific interactions have been observed and characterized between corresponding sense–antisense peptide pairs in numerous studies.^{28–31} On the basis of the nucleotide sequence of AK from *S. paramamosain*, the antisense peptide of AK was obtained by translating its antisense strand of the nucleotide sequence.

Peptide Aptamers Screened by Molecular Docking. Overlapping antisense peptides were constructed combining the pepsin cutting sites and linear epitopes of AK. The program AutoDock 4.0 was used to screen peptide aptamers from antisense peptides.³² The crystal structure of AK from *S. paramamosain* determined by Yang et al. [Protein Data Bank (PDB) code 5ZHQ] was performed as the receptor in molecular docking.³³ As ligand molecules, the three-dimensional (3D) structures of the antisense peptides were adjusted to be the most stable before performing molecular docking according to the method described by Cosconati et al.³⁴ After a Lamarckian genetic algorithm was run for 50 times, the conformational structures of the complex AK and antisense peptides were ranked according to binding energy. PyMOL software (Delano Scientific LLC, San Carlos, CA, U.S.A.) was used to generate docking graphics, and Discovery Studio software was applied to create a diagram of the two-dimensional (2D) structure for analysis.

Analysis of the Inhibitory Effect of Peptide Aptamers. According to the results of the molecular docking experiment, peptide aptamers covering the linear epitopes of AK and not destroyed by

pepsin were commercially synthesized using organic solid-phase synthesis technology.

The immunobinding reactivity of the peptide aptamers was detected by inhibition ELISA using sera from crab-allergic patients in Table 1 as the serum pool. The coupling agent, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, carbodiimide, was added to enhance the combination of AK and the peptide aptamer. Before inhibition ELISA analysis, the concentration of control peptide “GPPGP” and peptide aptamers was 6 ng/ μ L, which had been explored as the most appropriate concentration. The peptides were incubated with rAK or mAKs for 1 h at room temperature, in which there was 200 ng of rAK or mAKs. The positive control was rAK or mAKs, and the negative control was the peptide aptamer without rAK or mAKs.

The human LAD2 mast cell degranulation assay was performed to further analyze the inhibition effect of peptide aptamers on rAK and mAKs using sera from crab-allergic patients according to the method of Fu et al.³⁵ The positive control was rAK and mAKs; the negative control was PBS; and the control peptide was GPPGP.

Statistical Analysis. Data from the experiments are presented as the mean $\pm$ standard deviation (SD). Data was analyzed using a general linear model and Duncan's analysis of variance (ANOVA) test. Differences between groups were considered significant when *p* values were <0.05.

RESULTS

Site-Directed Mutagenesis of AK from *S. paramamosain*. Sequencing showed that the length of the open reading frame of the AK gene was 1071 bp, which encoded 356 amino acids.⁷ As reported, K₃₃ and T₁₇₄ were critical amino acids of conformational epitopes and C₂₀₁ formed an intramolecular disulfide bond with C₂₇₁.¹⁵ The selected amino acids were successfully replaced with Ala residues. Three variants were generated: mAK1 (AK/C_{201A}) contained C₂₀₁ mutated of the disulfide bond; mAK2 (AK/K_{33A}-T_{174A}) contained two critical amino acids mutated; and mAK3 (AK/T_{174A}-C_{201A}) contained one critical amino acid and disulfide bond mutated.

mAKs were analyzed by SDS–PAGE and western blot (Figure 1). The molecular mass of mAKs were about 40 kDa, and different from rAK that was expressed as a soluble protein, mAKs were expressed as inclusion bodies after inducing by IPTG. Western blot analysis demonstrated that mAKs reacted specifically with the rabbit anti-*S. paramamosain* AK polyclonal antibody. Then, mAKs were refolded by sequential dialysis until no urea remained, and they were purified as a single band using Ni–NTA resin (panels A–C of Figure 2).

Disulfide Bond and IgE Immunoreactivity Analysis of the Variant. To detect whether the disulfide bond of AK was broken after site-directed mutagenesis, the fluorescent reagent mBBR was used to selectively label the disulfide bonds. The more disulfide bonds a protein has the stronger the fluorescence intensity detected in the gel. Shown in Figure 2D, the fluorescence intensities of mAK1 and mAK3 were weaker than those of rAK and mAK2, confirming that the intramolecular disulfide bonds of mAK1 and mAK3 were destroyed.

Dot blot analysis was performed to detect the IgE binding activity of rAK and mAKs. The intensity of the dots shown on nitrocellulose membranes was quantified and shown as a histogram (Figure 2E). The immunoreactivity of mAK1, mAK2, and mAK3 decreased by 54.2 ± 4.6 , 40.1 ± 4.4 , and $71.4 \pm 3.7\%$, respectively. The results of the IgE inhibition ELISA (Figure 2F) showed that the inhibition rates of mAK1,

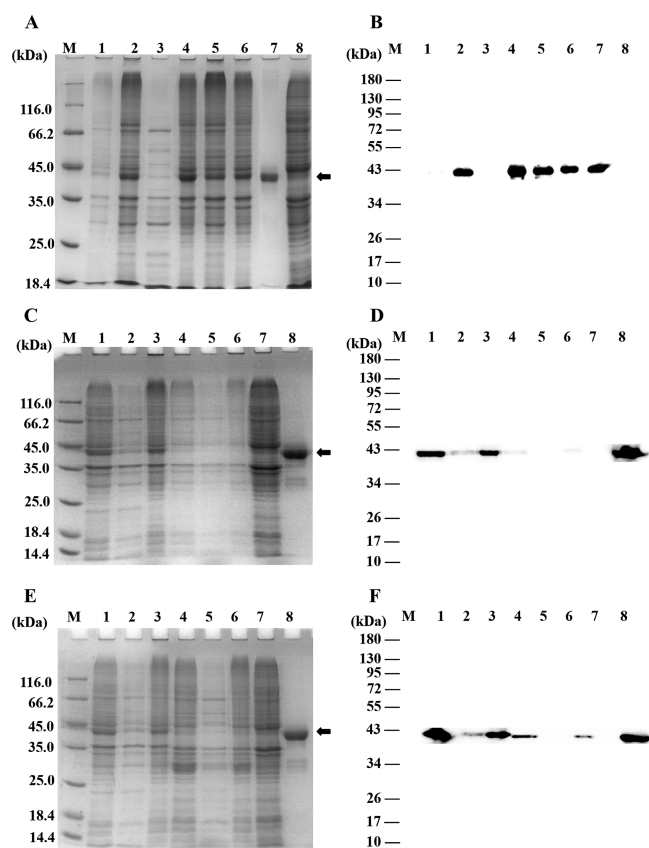


Figure 1. Recombinant expression of mAK from *S. paramamosain* in *E. coli*. (A) SDS–PAGE analysis of expressed mAK1 in *E. coli*. Lane M, protein marker; lane 1, the ultrasonicated fluid of the bacterial strain not induced by IPTG; lane 2, the ultrasonicated fluid of the bacterial strain; lane 3, the ultrasonicated supernatants of the bacterial strain; lanes 4–6, the ultrasonicated sediment of different bacterial strains; lane 7, the purified rAK; and lane 8, empty vector of pET-28a. (B) Western blot analysis of expressed mAK1 using rabbit anti-AK polyclonal antibody. (C) SDS–PAGE analysis of expressed mAK2 in *E. coli*. Lane M, protein marker; lanes 1–3, the ultrasonicated fluid, ultrasonicated supernatants, and ultrasonicated sediment of one bacterial strain; lanes 4–6, the ultrasonicated fluid, ultrasonicated supernatants, and ultrasonicated sediment of the other bacterial strain; lane 7, empty vector of pET-28a; and lane 8, purified rAK. (D) Western blot analysis of expressed mAK2 using rabbit anti-AK polyclonal antibody. (E) SDS–PAGE analysis of expressed mAK3 in *E. coli*. Lane M, protein marker; lanes 1–3, the ultrasonicated fluid, ultrasonicated supernatants, and ultrasonicated sediment of one bacterial strain; lanes 4–6, the ultrasonicated fluid, ultrasonicated supernatants, and ultrasonicated sediment of the other bacterial strain; lane 7, empty vector of pET-28a; and lane 8, purified rAK. (F) Western blot analysis of expressed mAK3 using rabbit anti-AK polyclonal antibody.

mAK2, and mAK3 on rAK were 60.5 ± 4.2 , 71.3 ± 3.6 , and $44.6 \pm 3.5\%$, respectively.

CD Spectra and Surface Hydrophobicity Analysis. To analyze whether the secondary structure of AK was altered by mutation or not, the CD spectra of rAK and mAKs were measured. As observed from the results (Figure 3A), rAK and mAKs had a positive maximum value at 190 nm and two negative maximum values at 208 and 222 nm, indicating that they had a predominantly α -helical secondary structure. However, the α -helix content of rAK and mAKs was clearly different. In comparison to rAK, the α -helix content in the

secondary structure of mAKs was significantly less. Conversely, there were more other secondary structures, including β -sheets, β -turns, and random coils. The secondary structure of mAK3 changed most significantly, and in comparison to rAK, the α -helix content was 22.7% less, the β -sheet content was 13.0% more, and the random coil content was 17.8% greater.

Moreover, the effects of the temperature on the secondary structure of rAK and mAKs were evaluated, and the thermal denaturation temperature of the proteins was measured using Global 3.0 software (panels B–E of Figure 3). As the temperature increased from 20 to 100 °C, the secondary structures of rAK and mAKs changed. The denaturation temperatures of rAK, mAK1, mAK2, and mAK3 were 50.7 ± 0.3 , 51.5 ± 0.2 , 56.4 ± 0.3 , and 46.2 ± 0.2 °C, respectively. These results showed that the secondary structures of mAKs were different from that of rAK, especially for mAK3, whose secondary structure was the most different.

Surface hydrophobicity analysis was performed to analyze the tertiary structures of rAK and mAKs. In comparison to rAK, the intensity curve of mAK1 was similar, while mAK2 and mAK3 were different, especially for mAK3. The intensity peak for mAK3 showed a significant decrease, with a peak that reached about half the size of rAK (Figure 3F).

Pepsin Cutting Sites and Antisense Peptide Analysis.

Our previous study revealed that AK is degraded into peptides after gastric pepsin digestion but resists duodenal digestion.³⁶ To analyze whether the linear epitopes of AK are destroyed after gastric and duodenal digestion, the pepsin cleavage sites of AK were analyzed using the ExPASy peptide cutter program. AK has 62 pepsin cleavage sites (Figure 4), and pepsin preferentially cleaves at Phe, Tyr, Trp, Leu, Arg, Lys, Pro, and His residues. Four linear epitopes of AK (L-AK-1–L-AK-4) were affected by pepsin, which could be cut after sufficient digestion.

The antisense peptides of AK were translated on the basis of the antisense nucleotide sequence of AK from *S. paramamosain* (Figure 4). A total of 17 overlapping antisense peptides were designed according to the pepsin cutting sites and linear epitopes of AK (Table S1 of the Supporting Information).

Peptide Aptamers Screened by Molecular Docking.

The molecular docking technique was performed to predict the molecular interactions between AK and antisense peptides. On the basis of the program AutoDock 4.0, the lower the binding energy suggested the more stable the binding of the receptor and ligand.³² According to the negative binding energy, five peptide aptamers were obtained. The peptide aptamers interacted with amino acid residues in linear epitopes of AK and formed hydrogen bonds (Table 3 and Figures S1–S4 of the Supporting Information). Peptide aptamer 3 (HLGLN) had the lowest binding energy, taking the docking structure of AK and peptide aptamer 3 for example, and their 3D and 2D structures were shown in Figure 5. Peptide aptamer 3 interacted with amino acid residues of AK via hydrogen bonds, van der Waals, and π – π stacked. Among them, the hydrogen-bonding interaction played the most important role in binding peptide aptamer 3 with AK to stabilize the docking complex. Additionally, nine amino acid residues in the linear epitope of AK, Gly₁₃₃, Tyr₁₃₄, Glu₁₄₄, Glu₁₄₇, Met₁₄₈, Ser₁₅₀, Lys₁₅₁, Ser₁₅₄, and Thr₁₅₅, were detected as interacting with peptide aptamer 3.

Analysis of the Inhibitory Effect of Peptide Aptamers.

The five peptide aptamers that had negative binding energy by molecular docking were synthesized, and their immunobinding

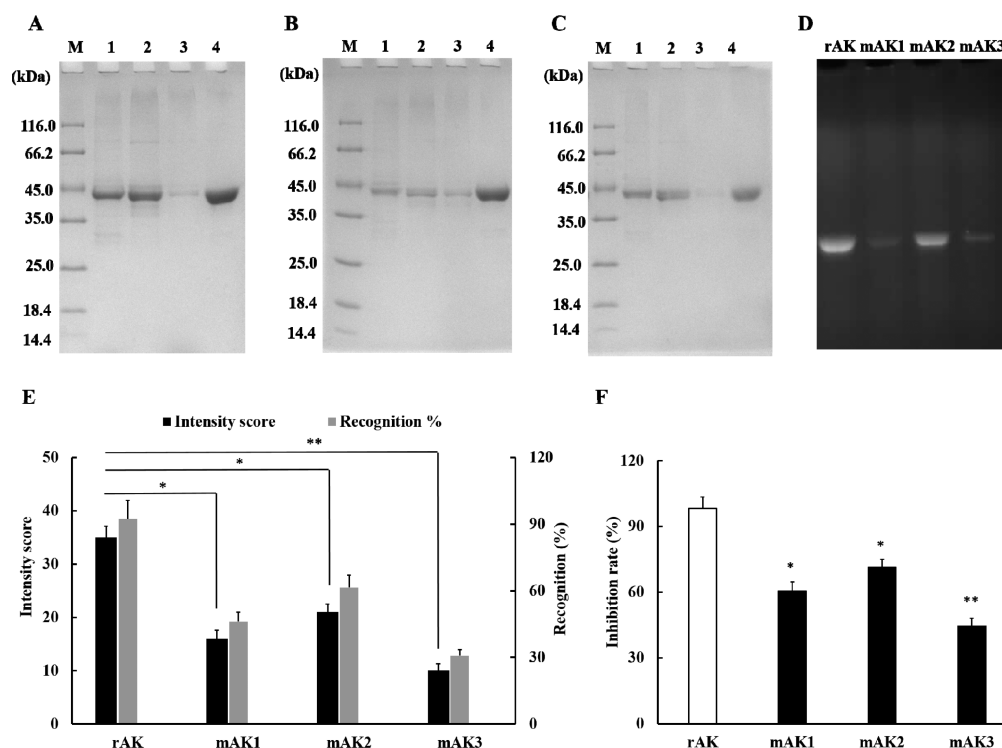


Figure 2. Purification, disulfide bond, and IgE immunoreactivity analysis of mAKs. (A) SDS–PAGE analysis of purified mAK1 using Ni–NTA. Lane M, protein marker; lane 1, the sample before purification; lanes 2 and 3, the eluted fractions; and lane 4, purified rAK. (B) SDS–PAGE analysis of purified mAK2 using Ni–NTA. Lane M, protein marker; lane 1, the sample before purification; lanes 2 and 3, the eluted fractions; and lane 4, purified rAK. (C) SDS–PAGE analysis of purified mAK3 using Ni–NTA. Lane M, protein marker; lane 1, the sample before purification; lanes 2 and 3, the eluted fractions; and lane 4, purified rAK. (D) Disulfide bond analysis of rAK and mAKs. Lane 1, purified rAK; lane 2, purified mAK1; lane 3, purified mAK2; and lane 4, purified mAK3. (E) IgE-binding activity analysis of rAK and mAKs by dot blot using crab-allergic patient serum. According to the intensity of the dots shown on the nitrocellulose membranes, the strong, middle, and weak intensities of the dots were calculated as scores of 3, 2, and 1 by three individuals. The dot blot results were quantized and shown as a histogram. (F) IgE-binding activity analysis of mAKs by the inhibition ELISA using the serum pool. rAK was coated on the solid phase, and the inhibitors were rAK or mAKs. The crab-allergic patient sera in Table 1 were used as the serum pool.

reactivity was detected by the IgE inhibition ELISA using sera from crab-allergic patients. As shown from Figure 6, peptide aptamers were able to significantly inhibit IgE-binding activity of rAK and mAKs. Among them, peptide aptamer 3 was slightly better than the other peptide aptamers, with inhibition rates of 28.3 ± 2.5 , 25.2 ± 1.9 , 26.5 ± 2.3 , and $21.7 \pm 2.0\%$ on rAK, mAK1, mAK2, and mAK3, respectively.

The human LAD2 mast cells were applied to further study the inhibitory effect of peptide aptamers, and the β -hexosaminidase release of human LAD2 mast cells was measured as an indicator of mast cell degranulation. As shown from Figure 7, the peptide aptamers could significantly reduce the β -hexosaminidase release of human LAD2 mast cells with rAK and mAKs, and peptide aptamer 3 was better than the other four peptide aptamers.

DISCUSSION

Shellfish, particularly crabs, are able to cause a severe type I allergic reaction, which has a growing prevalence in Asian countries.³ Among shellfish allergens, AK is considered to be a major allergen and is found in the myosinogen of shellfish.⁶ Currently, with the development of molecular biology techniques, site-directed mutagenesis is increasingly applied to modify proteins, to alter their thermal stability, or to reduce allergenicity.^{37,38} Our previous study identified antigenic epitopes of AK from *S. paramamosain*.¹⁵ Four conformational epitopes, D₃A₄K₄₃M₁A₅T₄₉T₄₄I₇, L₃₁K₃₃V₃₅T₃₂E₁₁E₁₈F₁₄S₃₄D₃₇,

V₁₇₇G₁₇₂M₁₇₃D₁₇₆Q₁₇₈T₁₇₄L₁₈₁K₁₇₅L₁₈₇, and R₂₀₂L₁₇₀Y₂₀₃E₁₉₀P₂₀₅W₂₀₄L₁₈₇T₂₀₆Y₁₄₅ were identified with the program LocaPep, including four critical amino acids, D₃, K₃₃, T₁₇₄, and W₂₀₄. We selected K₃₃, T₁₇₄, and C₂₀₁ for site-directed mutagenesis. Because D₃ was at the N terminal of AK and W₂₀₄ was close to C₂₀₁, primers were difficult to design at these positions. Hence, in this study, three hypoallergenic variants of AK were generated by site-directed mutagenesis. mAKs are expressed as inclusion bodies, which are different from rAK expressed by Mao et al.⁷ Similarly, Gattis et al. mutated the active site C₂₇₁ of AK, which changed the catalytic activity from horseshoe crab, and that form was also expressed as inclusion bodies.⁹

The results of CD spectra and surface hydrophobicity analysis suggested that the structures of mAKs were altered in comparison to that of rAK. The changed denaturation temperatures of mAKs were possibly due to their expression as inclusion bodies, while rAK was expressed as a soluble protein.⁷ Landeta et al. summarized the formation of disulfide bonds in prokaryotic systems, discussing examples for understanding their biological basis.³⁹ In this study, the structures of mAK1 and mAK3 changed more significantly, especially for mAK3, which resulted in a sharper decrease of the denaturation temperature than that of mAK2. As previously reported, the intramolecular disulfide bond formed between C₂₀₁ and C₂₇₁ plays an important role in maintaining the structure of AK.^{15,40} Our study also demonstrated that this

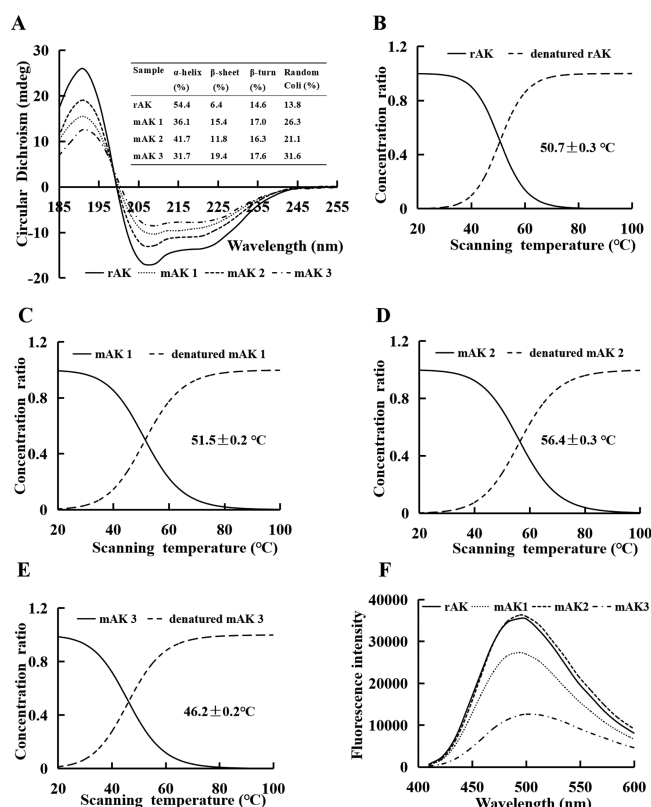


Figure 3. CD spectra and surface hydrophobicity analysis of mAKs. (A) Secondary structure analyzed at room temperature. (B, C, D, and E) Denaturation temperatures of rAK, mAK1, mAK2, and mAK3, respectively. (F) Surface hydrophobicity analysis of rAK and mAK.

Arginine kinase	MADAATIAKLEEGFKLEAATDCKSLKKYLTKSVFDQK	40
Antisense peptide	HSVSSGNGLQLLAELLQLSGSVALREQLLVEGLRHEVLEL	
Linear epitope		
Arginine kinase	GKKTSLGATLLDVIQSVENLDSGVGVYAPDAEAYTLFAPL	80
Antisense peptide	ALLGAQAGGEEIHDLGNLVQRANTHVSRLVGLVGVEEGQR	
Linear epitope		
Arginine kinase	FDPIHEDYHKGFKQTDKHPNKDFGDVNFQVNVDPDGKFLVI	120
Antisense peptide	EVGDDLVLVLTLLSVLVRVLEAIDVLEDVHVHGLALEHD	
Linear epitope	L-AK-1	
Arginine kinase	STRVRGGRSMGYPFNCLTEAQVKEMESKVSSTLNLG	160
Antisense peptide	GSAYTAAAGHLAVGEVGAELGLVLLHLGLNGGGQGVLT	
Linear epitope	L-AK-2 L-AK-3	
Arginine kinase	ELKGTYYPLTGMTKDQVQKLIIDHFLKEGDRFLQAAAC	200
Antisense peptide	LELACVIRESAHGLIHLQLQDVVVEELLTVAEQLGGIGA	
Linear epitope		
Arginine kinase	RYWPTGRGIVHNDKTLVWCDEEDHLRIISMQMGGDLGQ	240
Antisense peptide	VAPGSATADVVVVLGEQHPAVFLIVEADDGHLHTTVQAL	
Linear epitope	L-AK-4	
Arginine kinase	VYRRLVSAVNEIEKRVFSSHHDRLGFLTFCTNLGTTVRA	280
Antisense peptide	NVAAKDASNVLLLAHGEVGMVAQAEAGEAGVKGASHTG	
Linear epitope		
Arginine kinase	SVHIKLPKLAANREKLEEVAGKYSLVQRTRGEHTAEAGG	320
Antisense peptide	GDVDEGLQGGAFLKLFDSALVAELNAAGAAVLVSLTA	
Linear epitope		
Arginine kinase	VYDISNKRMRGLTEFQAVKEMQDGHLELIKIEKEMQ	356
Antisense peptide	HVIDGVLAHTKSFELNLLHLVTDKLEDLNLFLHL	
Linear epitope		

Figure 4. Analysis of pepsin cutting sites and sequence alignment of AK. The red colored letters are the predicted pepsin-cutting sites. The shaded letters are the linear epitopes. The antisense peptides were translated on the basis of the antisense nucleotide sequence of AK from *S. paramamosain*.

intramolecular disulfide bond was essential for structural stability of AK.

Furthermore, a previous study identified that the predominant epitope type in AK from *S. paramamosain* is the conformational epitope.^{7,15} The allergenicity could be reduced by mutating the amino acids of epitopes.^{38,41} In this study, the IgE-binding activity of mAKs showed a significant decrease compared to that of rAK. In combination of the analysis of structures, the change of the conformational structure had a significant effect on the immunobinding reactivity of AK. In addition, mutating critical amino acids in conformational epitopes of AK was able to reduce its allergenicity, revealing that these critical amino acids are important for mediating the interaction between epitopes and antibodies.

Food is subjected to gastric and duodenal digestion after consumption. AK was stable during intestinal digestion and was easily degraded by gastric digestion, a stage at which the degraded small peptides still contained immunobinding reactivity.³⁶ Moreover, four linear epitopes (L-AK-1:113–127, L-AK-2:127–141, L-AK-3:141–155, and L-AK-4:204–218) of AK from *S. paramamosain* were identified by the synthetic overlapping peptides.¹⁵ Thus, sequence analysis was performed between the linear epitopes and predicted sites, to further assess why the digested peptides still had immunobinding reactivity. Four linear epitopes of AK were destroyed after pepsin digestion, and the digested peptides retained more than four consecutive amino acid sequences of the linear epitope, which still could cause an allergic reaction.

Aptamers bind with target molecules specifically, a property that can be applied to perform different functions. Appert et al. screened a peptide aptamer that specifically bound with the second LIM domain of the LMO2 protein and disrupted its function.⁴² A DNA aptamer targeting the IgE epitope of the Asp f 1 allergen was selected, and the affinity between them was nanomolar, which brings about more accurate measurements.¹⁷ Another study gathered statistics of the interaction theories between sense and antisense peptides, which were experimentally verified in over 50 ligand–receptor systems.⁴³ In the present study, the peptide aptamers were able to interact with residues of AK linear epitopes and the intermolecular forces between them made the combinations more stable. Thus, peptide aptamers could inhibit the binding of AK with specific antibodies. In addition, the inhibitory effect of five peptide aptamers on rAK was a little better than that of mAK. The reason might be that rAK had higher allergenicity than that of mAKs and the inhibitory effect of its peptide aptamers was more distinct. Among these five peptide aptamers, peptide aptamer 3 had a slightly higher inhibition rate than the others, which might be due to having a lower binding energy and interacting with more residues of the AK linear epitope. Conversely, Root-Bernstein et al. reported the increased antigenicity of complexes formed by complementary proteins and peptides⁴⁴ as a result of the complexes altered antigen processing, protecting the components against proteolysis, altering fragmentation, and presenting novel antigens to the immune system. On the contrary, in the present study, after sufficient pepsin digestion, the peptide aptamers bound with the partial linear epitopes of AK specifically, blocking the recognition between the epitopes and antibodies, which reduced the allergenicity of AK.

Additionally, mast cells are important effector cells in allergic reactions, and the mediators released are the indicator of degranulation by mast cells to evaluate the strength of allergic reaction.⁴⁵ The human LAD2 mast cell degranulation assay was performed to further analyze the inhibition effect of

Table 3. Results of Molecular Docking between AK and Peptide Aptamer

name	sequence	binding energy (kcal/mol)	involved interactions with residues of the AK linear epitope	residues forming a hydrogen bond of AK ^a
P1	HDGSA	−2.32	Asp ₁₁₆ Gly ₁₁₇ Lys ₁₁₈ Val ₁₂₀ Ile ₁₂₁	Gly ₁₁₇ Val ₁₂₀ Lys ₂₈₆ Glu ₃₁₉
P2	GSAYT	−0.66	Ile ₁₂₁ Ser ₁₂₂ Arg ₁₂₄	Ser ₁₂₂ Ser ₂₈₂ His ₂₈₄
P3	HLGLN	−4.72	Gly ₁₃₃ Tyr ₁₃₄ Glu ₁₄₄ Glu ₁₄₇ Met ₁₄₈ Ser ₁₅₀ Lys ₁₅₁ Ser ₁₅₄ Thr ₁₅₅	Glu ₁₄₇ Ser ₁₅₀ Lys ₁₅₁ Ser ₁₅₄ Arg ₂₅₆
P4	GLNGG	−3.52	Tyr ₁₃₄ Glu ₁₄₇ Met ₁₄₈ Ser ₁₅₀ Lys ₁₅₁ Ser ₁₅₃ Ser ₁₅₄	Ser ₁₅₀ Lys ₁₅₁ Ser ₁₅₄
P5	SATAD	−0.71	Glu ₁₄₂ Lys ₁₄₆ Glu ₁₄₉ Trp ₂₀₄ Pro ₂₀₅ Thr ₂₀₆ Gly ₂₀₇	Lys ₁₄₆ Glu ₁₄₉ Leu ₁₇₀ Trp ₂₀₄

^aThe bold types were the residues of AK linear epitopes.

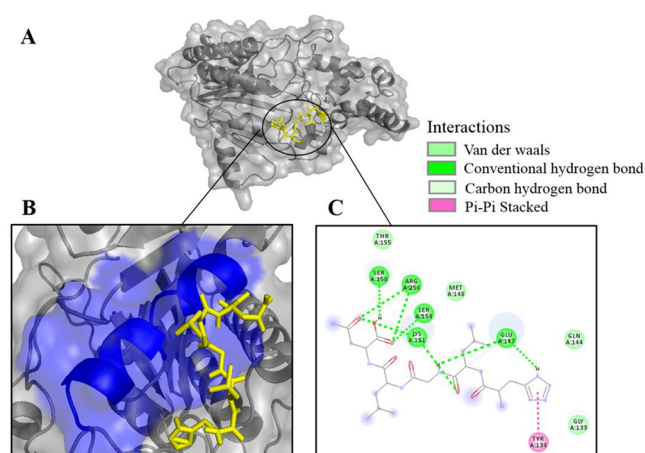


Figure 5. 3D and 2D structural analysis of AK docking with the peptide aptamer HLGLN. (A) Overall 3D diagram of the docking results. (B) Local enlarged view of the docking results. The blue color is the third linear epitope of AK, and the yellow color represents the peptide HLGLN. (C) 2D diagram of the docking results.

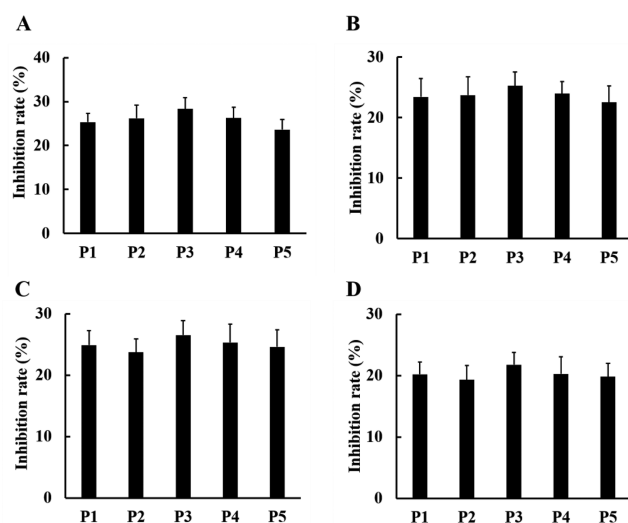


Figure 6. Analysis of the inhibitory effect of peptide aptamers on rAK and mAKs. (A–D) Inhibition rate of peptide aptamers on rAK, mAK1, mAK2, and mAK3, respectively. The positive control was rAK or mAKs, and the negative control was the peptide aptamer without rAK or mAKs. The crab-allergic patient sera in Table 1 were used as the serum pool.

peptide aptamers on rAK and mAKs *in vitro*. The peptide aptamers were able to significantly inhibit the β -hexosaminidase release of human LAD2 mast cells, suggesting that the peptide aptamers could inhibit the epitope of rAK and mAK binding with the specific IgE antibodies, which effectively reduce the degranulation of mast cells. The consistent results

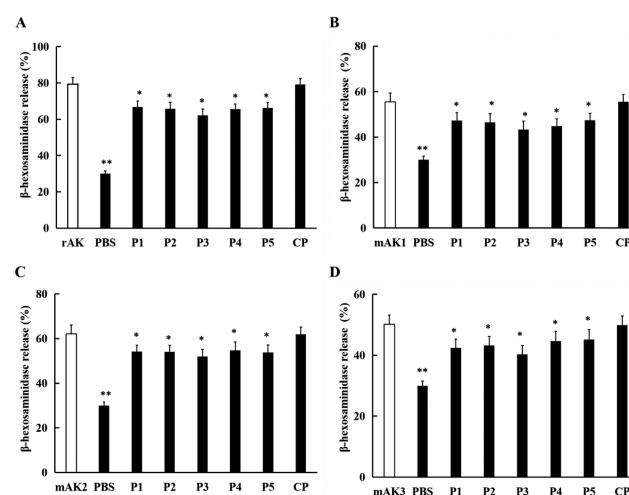


Figure 7. Human LAD2 mast cell degranulation induced by rAK and mAK with peptide aptamers. (A, B, C, and D) Measurement of β -hexosaminidase release of human LAD2 mast cells induced by rAK, mAK1, mAK2, and mAK3, respectively. The positive control was rAK and mAKs; the negative control was PBS; and “CP” means control peptide.

of the IgE inhibition ELISA and human LAD2 mast cell degranulation assay confirmed that the peptide aptamers could inhibit the IgE-binding activity of rAK and mAKs. Analogously, Sun et al. screened an aptamer of orai1 that could inhibit the β -hexosaminidase release of human LAD2 mast cells.⁴⁶ In a previous study, the antigenicity of AK was reduced by a processing method known as the Maillard reaction.⁴⁷ A better understanding of AK epitopes by modifying AK to reduce allergenicity could be used as a theoretical reference in food processing in further studies. Moreover, the high specificity and affinity of peptide aptamers could be applied to detect allergens in food safety, making their detection more precise.

In summary, the conformational structure and epitopes of AK from *S. paramamosain* were modified to reduce allergenicity. Three hypoallergenic variants were generated by mutating cysteine residues that formed an intramolecular disulfide bond and critical amino acids in conformational epitopes of AK. Additionally, molecular docking was used to screen for peptide aptamers specifically bound to linear epitopes of AK. These peptide aptamers inhibited the immunoreactivity of rAK and mAKs. The synergistic effect detected in this study could be applied to reduce the allergenicity of other shellfish allergens.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b00608.

Results of AK docking with antisense peptide (Table S1) and 3D and 2D structural analysis of AK docking with peptide aptamers HDGSA (Figure S1), GSAYT (Figure S2), GLNGG (Figure S3), and SATAD (Figure S4) (PDF)

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

AK, arginine kinase; ANS, 8-anilino-1-naphthalenesulfonate; CD, circular dichroism; CP, control peptide; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; IgE, immunoglobulin E; IgG, immunoglobulin G; IPTG, isopropyl β -D-1-thiogalactopyranoside; mAK, mutant arginine kinase; PBS, phosphate-buffered saline; rAK, recombinant arginine kinase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; 2D, two-dimensional; 3D, three-dimensional

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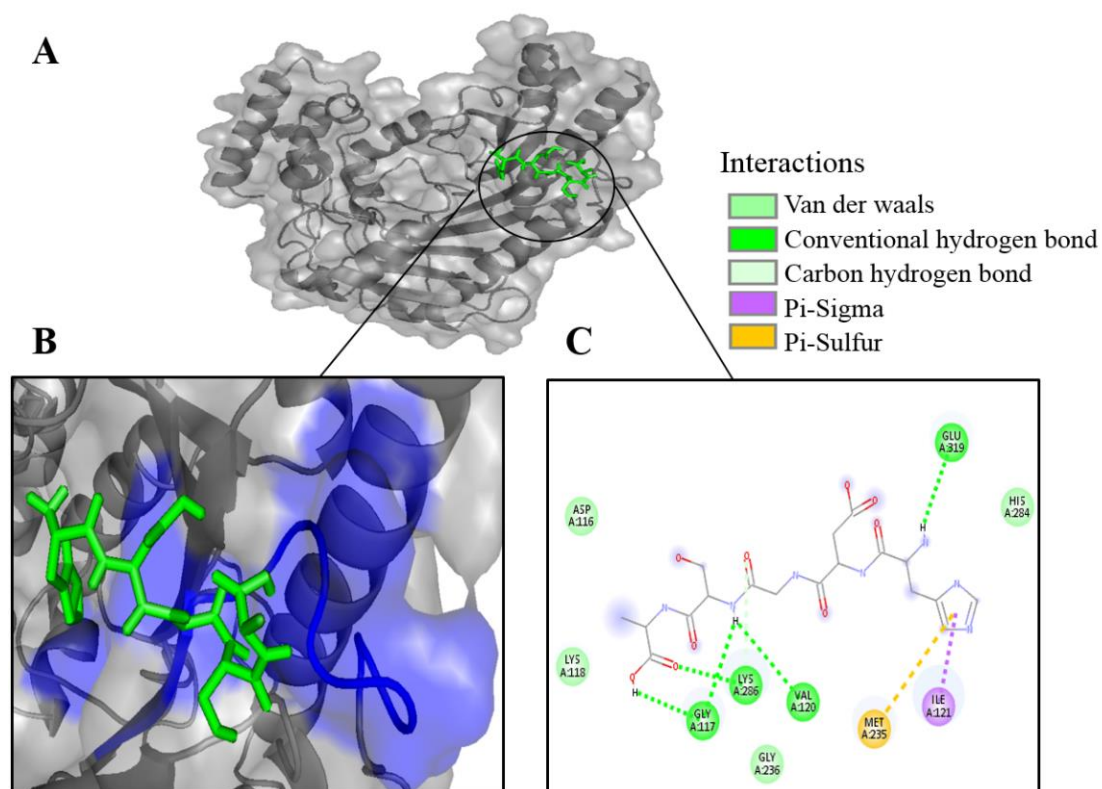
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Table S1. The results of AK docking with antisense peptide.

Antisense peptide ^a	Position	Binding energy (kcal/mol)
VGIA	113-116	2.16
HDGSA	119-123	-2.32
DGSAY	120-124	13.15
GSAYT	121-125	-0.66
SAYTA	122-126	3.22
AYTAA	123-127	15.39
AGHLA	128-132	6.01
GHLAV	129-133	34.68
EVGA	135-138	8.21
LLHLG	145-149	12.17
LHLGL	146-150	0.62
HLGLN	147-151	-4.72
LGLNG	148-152	3.13
GLNGG	149-153	-3.52
GSATA	204-208	7.86
SATAD	205-209	-0.71
VVVVL	211-215	0.12

^a The bold types were the antisense peptide screened as peptide aptamers.

Figure S1



The 3D and 2D structural analysis of AK docking with peptide aptamer HDGSA.

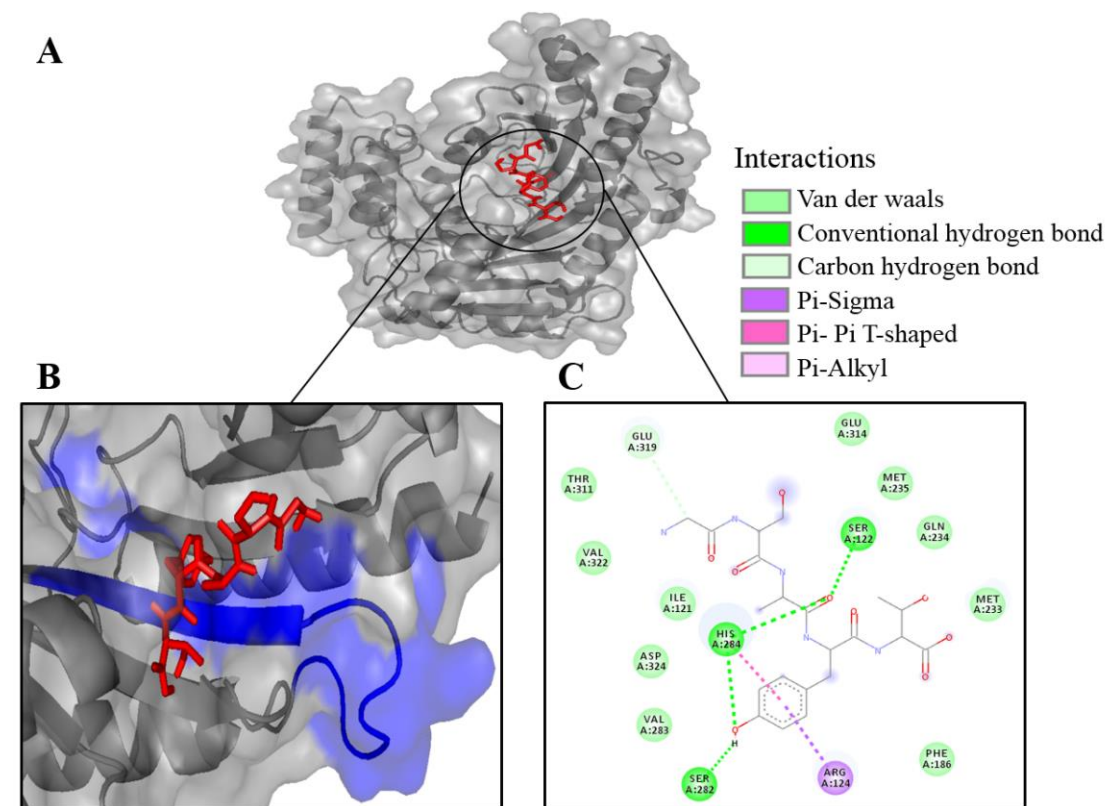
A, Overall 3D diagram of docking results.

B, Local enlarged view of docking results.

The blue color mean the first linear epitope of AK, the green color mean peptide HDGSA.

C, 2D diagram of docking results.

Figure S2



The 3D and 2D structural analysis of AK docking with peptide aptamer GSAYT.

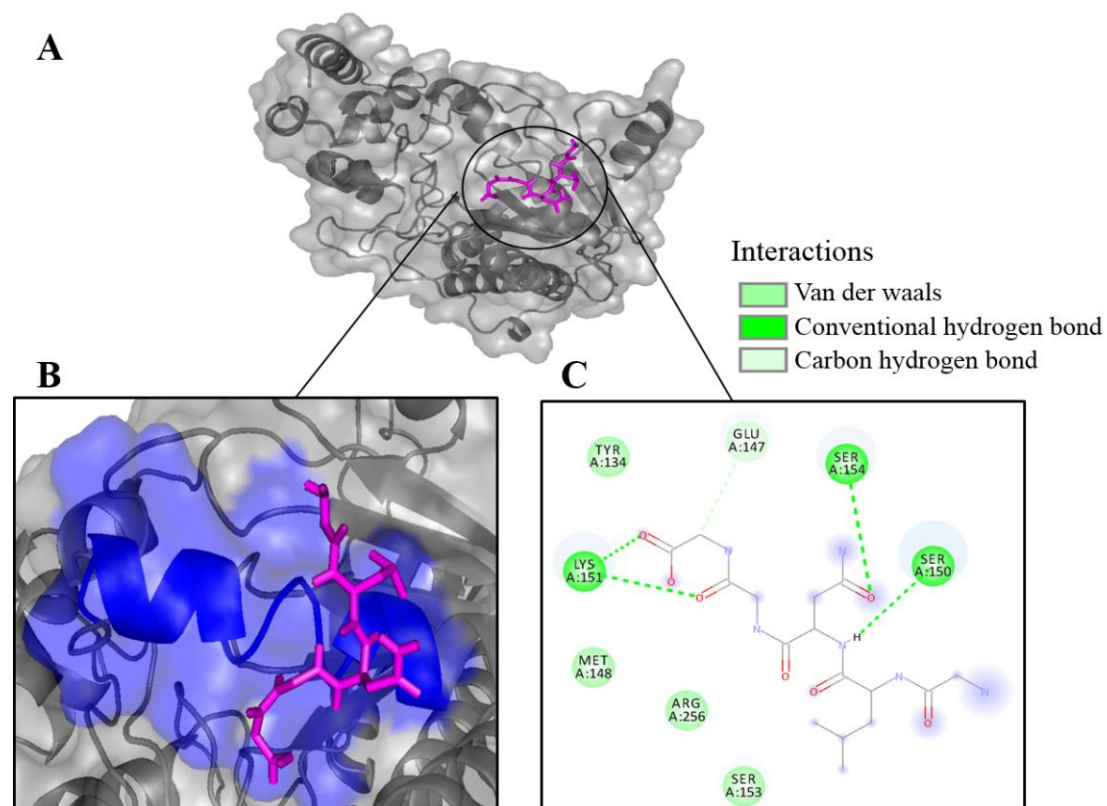
A, Overall 3D diagram of docking results.

B, Local enlarged view of docking results.

The blue color mean the first linear epitope of AK, the red color mean peptide GSAYT.

C, 2D diagram of docking results.

Figure S3



The 3D and 2D structural analysis of AK docking with peptide aptamer GLNGG.

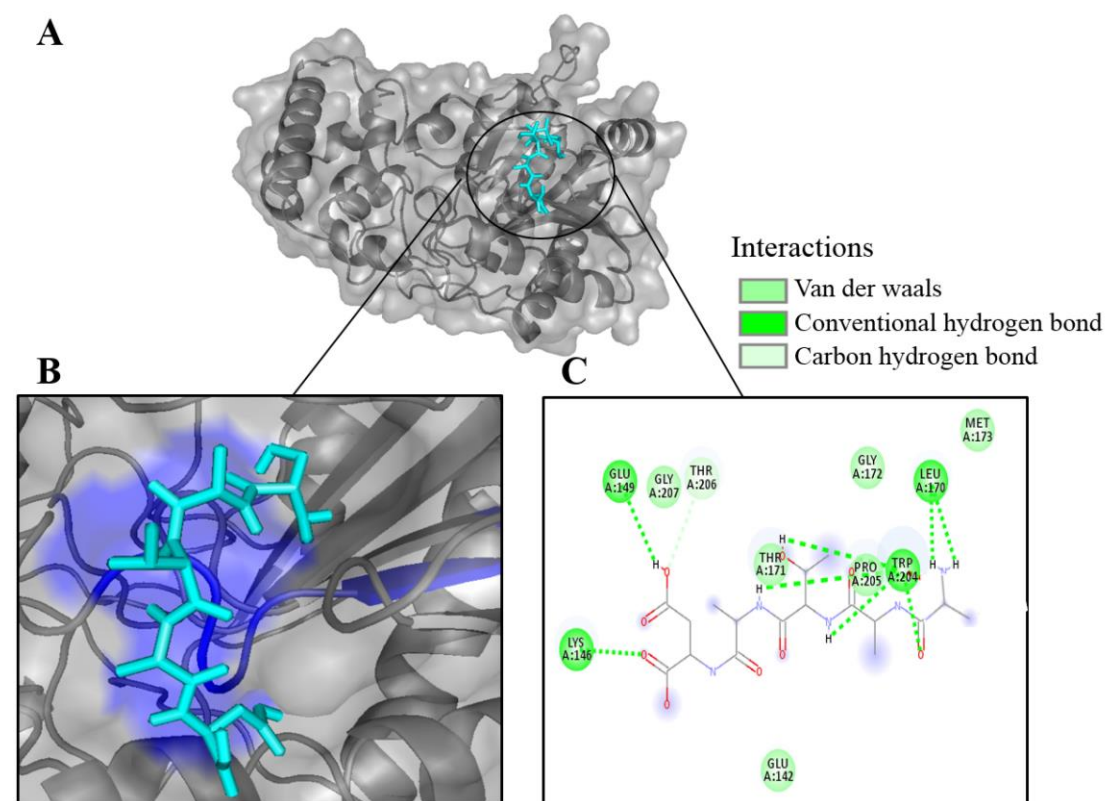
A, Overall 3D diagram of docking results.

B, Local enlarged view of docking results.

The blue color mean the third linear epitope of AK, the pink color mean peptide GLNGG.

C, 2D diagram of docking results.

Figure S4



The 3D and 2D structural analysis of AK docking with peptide aptamer SATAD.

A, Overall 3D diagram of docking results.

B, Local enlarged view of docking results

The blue color mean the fourth linear epitope of AK, the light blue color mean peptide SATAD.

C, 2D diagram of docking results.