

RESEARCH ARTICLE

Design and Stability Evaluation of Active Peptides from Indonesian Echinozoa as Acetylcholinesterase (AChE) Inhibitors for Alzheimer's Therapy

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Abstract

BACKGROUND: Alzheimer's disease is characterized by cognitive decline resulting from decreased acetylcholine (ACh) levels due to excessive acetylcholinesterase (AChE) activity. Current therapies, such as galantamine, have several side effects. Bioactive peptides derived from marine Echinozoa (sea urchins and sea cucumbers) have emerged as promising therapeutic agents owing to their structural diversity and diverse bioactivities. Previous studies identified peptides from sea cucumbers and sea urchins collected along the southern coast of Gunung Kidul, Yogyakarta (VLCAGDLR, SWIGLK, MNGKKITVRPR, and KTKDLK), which exhibit acetylcholinesterase (AChE) inhibitory activity. However, the therapeutic use of these peptides is challenged by blood-brain barrier (BBB) penetration and stability issues. Therefore, this study was conducted to identify candidate peptides through *in silico* analysis and to evaluate their stability in phosphate-buffered saline (PBS) as potential AChE inhibitors.

METHODS: Molecular docking was conducted to evaluate peptide binding affinity to the active site. The best candidate peptides were synthesized and tested *in vitro* for AChE inhibition using a colorimetric method. Stability was assessed in PBS by monitoring aggregation through turbidity and Congo Red assays.

RESULTS: The sea cucumber peptide SWIGLK showed strong binding affinity (−10.2 kcal/mol) and 12.11% inhibition at 0.19 mM, while the sea urchin peptide KTKDLK exhibited −8.2 kcal/mol and 11.50% inhibition at 0.19 mM. Both peptides remained stable in PBS without aggregation for up to 48 h.

CONCLUSION: SWIGLK and KTKDLK demonstrate the most significant AChE inhibitory activity and maintained structural stability, hence supporting their potential as peptide-based candidates for Alzheimer's therapy.

KEYWORDS: Alzheimer, AChE inhibitor, holothuroidea, echinoidea, bioactive peptide, peptide stability

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Introduction

More than 55 million people worldwide suffer from neurodegenerative diseases, with Alzheimer's disease accounting for 60–70% of these cases. Neurons play a crucial role in processing information and transmitting signals to target cells.(1) Damage to these neurons leads to symptoms such as memory loss, behavioral changes, and progressive

cognitive decline over time.(2) Alzheimer's therapies recommended by the Food and Drug Administration (FDA) include donepezil, rivastigmine, and galantamine, which act as cholinesterase inhibitors, as well as memantine, which acts as a non-competitive inhibitor. The use of these drugs still causes various side effects, including diarrhea, nausea, vomiting, dizziness, and muscle cramps, and is unable to halt the progression of the disease.(3) In recent decades, most clinical Alzheimer's disease drugs have failed to

be developed due to limited effectiveness or adverse side effects. FDA approval of several new types of drugs, as well as the long-term effectiveness and safety of these drugs, still need to be further validated.(4)

In Alzheimer's patients, damage to brain neurons causes the acetylcholinesterase (AChE) enzyme in the synaptic cleft to break down acetylcholine too quickly, disrupting communication between neurons and resulting in memory loss.(5,6) Therefore, it is necessary to treat Alzheimer's disease patients with therapy that restores cholinergic deficits, in this case through the AChE approach.(7) Research has developed an Alzheimer's drug compound that can inhibit the aggregation of amyloid-beta ($A\beta$), AChE, and beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) derived from the breakdown of heterocyclic amines, and showed significant inhibitory activity in enzyme inhibition.(8)

Marine organisms from the Echinozoa class (such as sea urchins and sea cucumbers) produce various bioactive peptides with broad therapeutic potential, including antioxidant, anticancer, anti-fatigue, antidiabetic, antihypertensive, antimicrobial, and memory-enhancing activities through various molecular pathways that is related to the regulation of oxidative stress, energy metabolism, and nerve function.(9) Peptides are able to efficiently cross the blood-brain barrier, reaching the affected areas of the brain to provide therapeutic effects.(10) In general, peptides have low toxicity and can be modified to increase their stability and resistance to degradation. Peptides can modulate various molecular targets, such as enzymes, inflammatory pathways, and synaptic function, thereby potentially addressing various aspects of Alzheimer's pathology.(11)

In targeting AChE for treatment, the ability of biomolecules to cross the BBB must be considered. The main obstacle currently being focused on is the failure of drug delivery to penetrate the blood brain barrier (BBB). A total of 98% of biomolecular drugs cannot penetrate the BBB, and 100% of biological drugs cannot penetrate the BBB.(12) Echinozoa peptides have the potential to be used as therapeutic agents due to their diversity and effectiveness in treating neurodegenerative diseases, including targeting the AChE enzyme.(13) Bioactive peptides have great potential as therapeutic agents because most bodily functions result from amino acid interactions and they have fewer side effects compared to large proteins.(14) Peptide-based inhibitors have several advantages, including high specificity, low toxicity, rapid clearance, and the potential for de novo design to precisely tailor their properties. (15) Additionally, the diversity of side-chain structures

in peptides allows for selective recognition of various molecular targets, more controlled duration of action, and more consistent therapeutic effects.(16) The stability and efficacy of peptides must be maintained during drug delivery. Peptide instability can lead to peptide inactivation, thereby reducing therapeutic effects.(17)

Previous research has explored active peptides from the Echinozoa (*Stomopneustes variolaris* and *Holothuria pardalis*) originating from sea cucumbers and sea urchin gonads on the southern coast of Gunung Kidul, Yogyakarta. These peptides have been identified as potential candidates and are to be tested for their activity in inhibiting acetylcholinesterase (AChE) enzymes *in vitro*. Previous research has predominantly concentrated on identifying peptide sequences and evaluating their initial inhibitory effects on AChE, without addressing their stability or the underlying molecular mechanisms. In contrast, the present study not only examines the AChE inhibitory potential of peptides derived from Echinozoa but also emphasizes their stability through both computational and experimental analyses. Given that peptide instability and degradation can significantly diminish therapeutic efficacy by limiting their ability to reach the target site, it is essential to develop innovative strategies to enhance peptide stability. However, studies specifically focusing on this stability and inhibitory potential of Echinozoa-derived peptides against AChE remain limited. Based on the *in silico* results, parent peptides were selected and the lowest binding affinity values, resulting in four candidate parent peptides for further *in vitro* testing. Therefore, the present study was conducted to design and evaluate the stability of active peptides from Echinozoa as potential AChE inhibitors for Alzheimer's therapy.

Methods

Enzyme Inhibitory Assay of AChE

The inhibitory activity of the peptides against AChE was evaluated using a colorimetric method based on the Acetylcholinesterase Inhibitor Screening Kit MAK324 (Sigma-Aldrich, St. Louis, MO, USA). Four-hundred U/L Acetylcholinesterase solution (Cat. No. C3389; Sigma-Aldrich), peptides (purity >95%, WuHan Dangang Biological Technology, Wuhan, China), and galantamine hydrobromide (Cat. No. G1660; Sigma-Aldrich) were prepared prior to the assay. In a 96-well plate, 5 μ L of 40% dimethyl sulfoxide (DMSO) and 45 μ L of assay buffer were added for the no-enzyme control, whereas 5 μ L of 40% DMSO and 45 μ L of AChE were added for the no inhibitor

control. For the test wells, 5 μ L of peptide or galantamine was mixed with 45 μ L of AChE solution and incubated for 15 min. A reaction mixture containing 154 μ L of assay buffer, 1 μ L of substrate, and 0.5 μ L of 5,5'-Dithiobis-2-nitrobenzoic acid (DTNB) was prepared, and 150 μ L of this mixture was added to each well. Absorbance was measured at 412 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Allsheng FlexA-200, Allsheng, Hangzhou, China) at 0 and 10 min. The percentage inhibition was calculated using the following formula: Inhibition (%) = $(1 - (\Delta A \text{ Test Cpd}) / (\Delta A \text{ no inhibitor})) \times 100$; with ΔA test cpd = difference in absorbance of the test compound, and ΔA no inhibitor = difference in absorbance of the inhibitor-free control.

***in silico* Analysis and Molecular Docking**

Three-dimensional models of the peptides as ligand were generated using Chimera (<https://www.cgl.ucsf.edu/chimera/>) by looking at the structure of amino acids forming a double helix or coil structure, while the human AChE structure (UniProt ID: P22303) was obtained from the Protein Data Bank (<http://rcsb.org/>) and modeled using SwissModel (<https://swissmodel.expasy.org/>). Active sites of AChE were predicted using PrankWeb (<https://prankweb.cz/>). Molecular docking simulations were performed using PyRx with both blind and rigid docking approaches at the binding site interacting with galantamine. The root mean square deviation (RMSD) values were calculated using PyMOL (<https://www.pymol.org/>) to evaluate conformational stability. Interactions between peptides and AChE residues were visualized with BIOVIA Discovery Studio (Dassault Systèmes, Vélizy-Villacoublay, France). Based on unfavorable interactions, peptides were truncated into 3–4 amino acid fragments, which were further docked against AChE using the same protocol to compare binding affinity and binding interactions with the parent peptides.

Peptide Aggregation and Stability in PBS

Phosphate-buffered saline (PBS) was prepared by dissolving 4 g NaCl, 0.72 g Na_2HPO_4 , 0.1 g KCl, and 0.12 g KH_2PO_4 in distilled water up to 500 mL. Peptides were dissolved at 1 mg/mL and diluted with PBS to a final volume of 100 μ L in 96-well plates. To evaluate concentration-dependent stability, peptides were prepared at 0.5, 0.75, 1, and 1.25 mg/mL in a final volume of 100 μ L with PBS. Absorbance was measured at 350 nm using Allsheng FlexA-200 ELISA reader with indirect principal at 0 h, 30-min, 1, 2.5, 2, 2.5, 3, 24, and 48 h.

Congo Red Assay for Aggregation Evaluation

A 20 μ M Congo Red solution was prepared from a 100 μ M stock by dissolving 1 mg of Congo Red in 14.36 mL of sterile distilled water. Peptide solutions were prepared to achieve a final concentration of 1 mg/mL in 100 μ L of assay volume. Peptide samples were mixed with PBS and measured at 490, 540, and 660 nm as baseline values. Subsequently, 20 μ L of Congo Red solution was added to each sample well and to 1.5 mL Eppendorf tubes (duplicate). Absorbance was re-measured at the same wavelengths at 0, 30, 60, 120, 180 min, and 22 h overnight. Morphological observations of peptide aggregates were performed using a light microscope at the same time points.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). Differences among groups were assessed using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test to compare each treatment group with the control. A p -value < 0.05 was considered statistically significant.

Results

***in silico* Analysis Results**

Molecular docking showed that galantamine bound strongly to the active site of AChE with a binding affinity of -8.6 kcal/mol. The interaction involved residues Tyr103, Asp105, Tyr155, Trp317, Ser324, Val325, Phe326, Arg327, Phe328, Tyr368, Phe369, and Tyr372 (Table 1). Among the test peptides, CP-1 formed a conventional hydrogen bond with Tyr372, an important residue at the peripheral anionic site (PAS) of AChE. CP-2 exhibited van der Waals interactions with Trp317, also located at the PAS, and Ser324, a component of the catalytic triad. UP-1 interacted with Tyr103 via a conventional hydrogen bond, while UP-2 interacted with Trp317 through van der Waals forces, both residues being essential at the PAS (Figure 1).

Peptide Inhibition Activity

The inhibition assay demonstrated that CP-1 had higher inhibitory activity (12.11% at 0.19 mM) compared with CP-2. Similarly, UP-1 showed stronger inhibition (25.27% at 0.19 mM) than UP-2 (Table 2). These findings were consistent with the molecular docking results, in which CP-1 and UP-1 exhibited more favorable (lower) binding affinity values compared with CP-2 and UP-2.

Table 1. Molecular docking results between AChE and galantamine (positive control) and peptides from Echinozoa.

Name of Peptide	Sequence of Peptide	Binding Affinity	RMSD	Amino Acid Peptide	Bond Type	Active Site Residue of AChE
Galantamine		-8.6	1.318		Carbon hydrogen	TYR155
					Carbon hydrogen	TYR368
					Carbon hydrogen	ASP105
					Pi-Sigma	TYR372
					Pi-Alkyl	TRP317
CP-1	SWIGLK	-10.2	0.740	SER1	Conventional hydrogen bond	TYR372
				TRP2	Conventional hydrogen bond	TRP317
				GLY4	Carbon hydrogen	TRP317
CP-2	VLCAGDLR	-6.7	0.723		Van der waals	SER324
UP-1	KTKDLK	-8.2	0.730	LYS1	Conventional hydrogen bond	GLN322
				THR2	Unfavorable bond	TRP317
				LYS3	Conventional hydrogen bond	TYR103
					Conventional hydrogen bond	TYR106
UP-2	MNGKKITVRPR	+17.3	0.746	MET1	Alkyl	LEU320
					conventional hydrogen bond	SER324
					unfavorable bond	LEU320
				ASN2	conventional hydrogen bond	HIS318

CP : Sea cucumber peptide; UP : Sea urchin peptide.

Peptide Aggregation and Stability

One-Way ANOVA analysis was used to determine the effect of time on peptide aggregation with 0 minutes as the control. Peptides were tested for aggregation in PBS buffer pH 7. CP-2 underwent aggregation due to significant differences at 24 and 48 hours (Figure 2B). CP-1 showed good stability up to 48 hours with no aggregation detected (Figure A). UP-1 in PBS pH 7 solution showed no significant difference in absorbance ($p>0.05$) in tests from 0 to 48 hours, indicating that the peptide is stable (Figure 2C). UP-2 showed no significant difference in absorbance ($p>0.05$) between 0 and 30 minutes up to 24 hours, but at 48 hours, the p -value was 0.027, indicating a significant difference ($p<0.05$) compared to 0 minutes. Based on these results, UP-2 showed instability within 48 hours (Figure 2D).

Figure 3 showed the stability of CP-1 was evaluated at concentrations ranging from 0.5 to 1.25 mg/mL over different incubation times. Two-way ANOVA revealed no significant effect of either time or concentration on CP-1 stability ($p>0.05$). UP-1 also exhibited no significant changes in absorbance across all tested concentrations ($p>0.05$). These results indicated that CP-1 and UP-1 remained stable without aggregation in the phosphate buffer at all tested concentrations, suggesting long-term physical stability.

Figure 4 and 5 present the Congo Red staining result observed under light microscopy. No aggregation was detected for CP-1 or CP-2, indicated by the absence of

red spots or clumps after 22 h of incubation with hourly monitoring and overnight observation. Similarly, UP-1 showed no red staining up to overnight incubation. In contrast, UP-2 exhibited peptide aggregation, as indicated by the presence of red-stained aggregates after overnight incubation.

Discussion

This study shows that peptides from sea cucumbers and sea urchins have significant ability to inhibit AChE enzyme activity at different levels. Sea cucumber peptide CP-1 has stronger activity because it is able to bind to the active site of the PAS enzyme based on binding affinity results. CP-2 has lower activity because the bond formed is limited to van der Waals bonds, resulting in a lower percentage of inhibition. The activity of sea urchin peptides shows that UP-1 has more significant activity than UP-2. This is in line with the lower binding affinity results and the presence of unfavorable bonds, indicating the existence of unwanted bonds. The binding affinity value (ΔG) is a parameter of conformational stability between ligands and macromolecules.(18) The binding affinity of peptides that do not bind to the target site is also lower than that of ligands that bind to important sites such as PAS.(19) Furthermore, RMSD values of 0 or less than 3 Å suggest that the peptide–AChE docking simulations possess a high

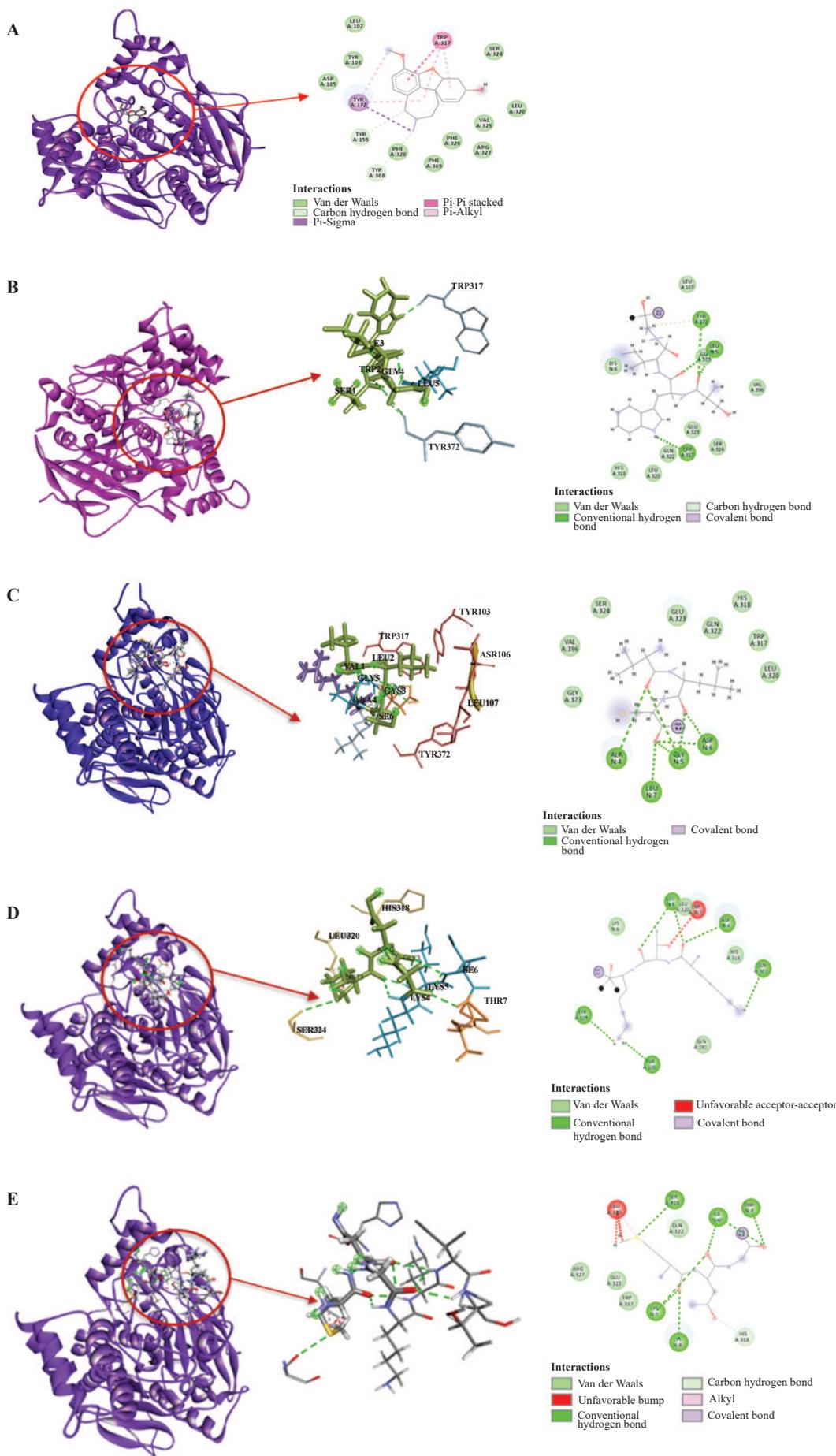


Figure 1. Visualization of molecular docking between peptides and AChE in 3D and 2D. A: The binding interaction of galantamine with AChE as the control ligand. B: The docking result of peptide CP-1 with AChE. C: The interaction of peptide CP-2 with AChE. The binding of peptide UP-1 with AChE. E: The docking of peptide UP-2 with AChE. The visualization highlights the orientation of each peptide in the PAS region of the enzyme, indicating their potential inhibitory activity.

Table 2. Peptide inhibition percentage results.

Sample	Concentration (mM)	Percent Inhibition
CP-1	0.19	12.11%
CP-2	0.80	35.22%
UP-1	0.19	25.27%
UP-2	0.76	5.03%

degree of reliability and predictive accuracy.(20) Each peptide has a different amino acid length and amino acid residue sequence. This illustrates how amino acid variation and sequence can influence the type of interaction and its inhibitory capacity. Additionally, if ligands form hydrogen bonds outside the PAS domain (outside the PAS binding pocket), these interactions will cause binding instability and reduce affinity.(21)

Binding affinity and RMSD values are important parameters in docking analysis, as both are used to assess the ability of ligands to interact with receptors and the stability of the complexes formed.(22) The use of molecular docking in this study is in line with the approach used in the study of *Bidens vulgaris* leaf essential oil, where docking simulations were used to predict affinity and inhibition potential against target enzymes. *Bidens vulgaris* leaf essential oil shows potential as a β -lactamase inhibitor based on molecular docking results with ΔG values ranging from -4.3 to -8.0 kcal/mol.(23) Low binding affinity values correlate with the ability of ligands to effectively inhibit protein function, confirming that docking analysis is a reliable approach for predicting the biological activity of drug candidates.(24)

Recent studies have highlighted the neuroprotective potential of marine-derived peptides against Alzheimer's disease. Sea cucumber peptides improved memory performance and repaired cholinergic function in scopolamine-induced memory-impaired mice by increasing ACh levels and protecting hippocampal neurons.(25) Similarly, other study identified nine novel oligopeptides (IGFH, LGFH, DWF, and FQF) from simulated gastrointestinal hydrolysates of sea cucumbers that exhibited significant CD38 inhibitory and anti-A β aggregation activities. These findings demonstrate the strong potential of sea cucumber peptides as natural agents for Alzheimer's prevention and therapy.(26) This reinforces the ability of sea cucumber peptides to overcome Alzheimer's disease through certain mechanisms. Pig bristles have potential in current treatments, including Alzheimer's, although studies on pig bristle peptides are still very limited. Previous research showed that Echinochrome A can inhibit AChE *in vitro* through an irreversible/uncompetitive mechanism and exhibits antioxidant activity, indicating the therapeutic potential of echinoids in the context of acetylcholine-related diseases.(27)

The stability of CP-1 and UP-1 peptides is the best, characterized by no significant difference from the control and remaining stable across variations in peptide concentration. This may be influenced by the shorter amino acid length, which gives both peptides good stability. Peptides with more than 5 consecutive amino acids tend to be at higher risk of aggregation, known as Aggregation-Prone Region (APR), which is the initial core of aggregate formation with high hydrophobicity and low net charge.

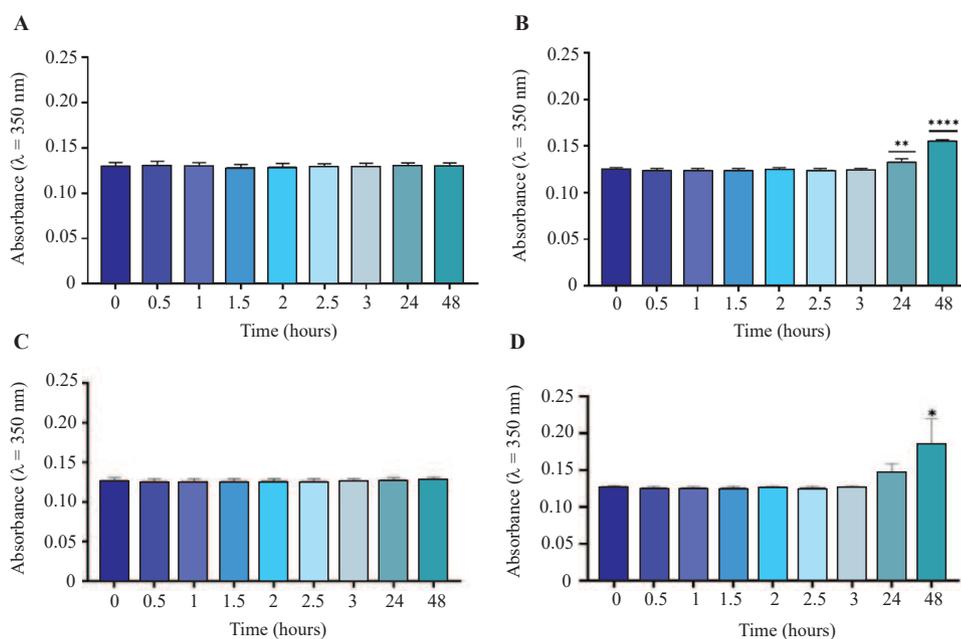


Figure 2. Results of parent peptide stability screening in PBS buffer at a concentration of 1 mg/mL. A: CP-1; B: CP-2; C: UP-1; D: UP-2. Results are presented as mean \pm SEM. Analysis was performed using One-Way ANOVA with comparison to the control group (0 minutes). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control (0 minutes). Stability was monitored at 0 h-48 h.

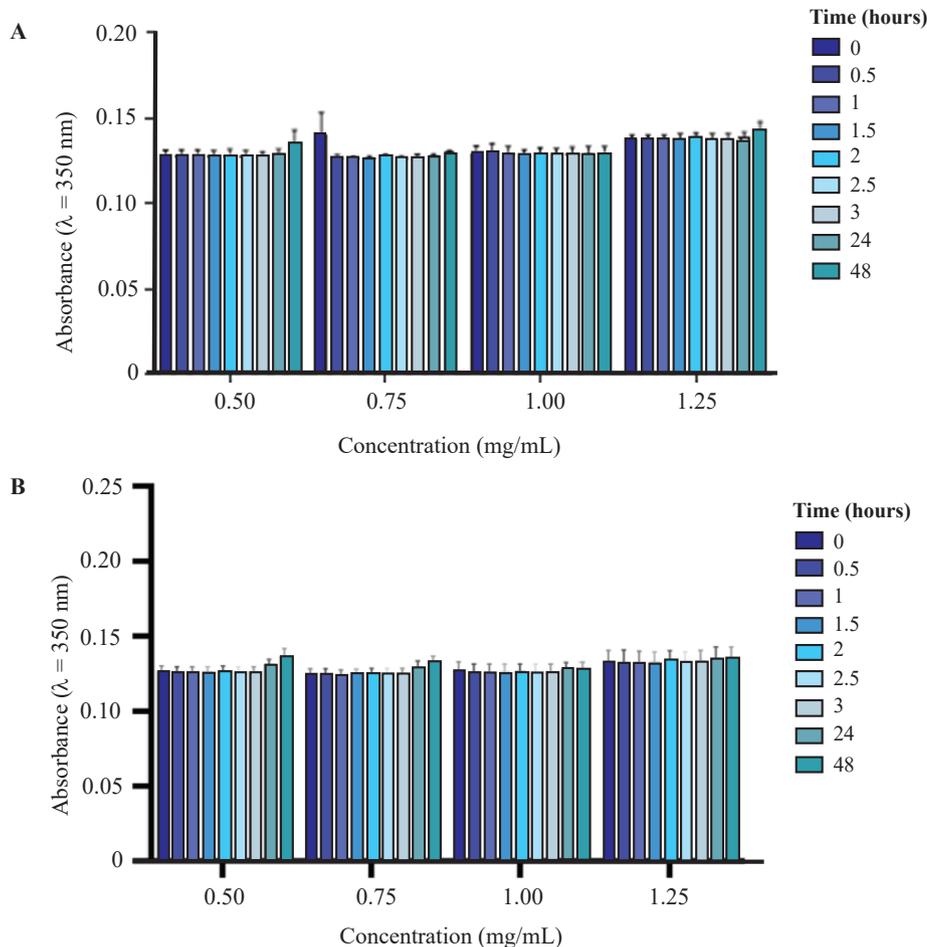


Figure 3. The stability of CP-1 and UP-1 was tested at concentrations of 0.5–1.25 mg/mL with varying times up to 48 h. A: CP-1; B: UP-1. Two-Way ANOVA analysis was used to determine the significant effect of time or concentration on the stability of CP-1 ($p>0.05$).

(28) Peptides with a hydrophobic amino acid sequence tend to have a higher probability of aggregation due to the possibility of attraction and the tendency to form spherical or ball-like aggregates to reduce the surface area of the aggregate in solution.(29) The aggregation process of the CIGB-814 peptide occurs in a gradual, multi-step manner and is significantly influenced by peptide concentration. At micromolar concentrations, the peptides aggregate to form small oligomers of nanoscale dimensions, primarily due to interactions between the hydrophobic regions of the peptide.(30)

This was further validated by Congo Red staining, which showed that peptides with longer amino acid sequences were more prone to aggregation. This was evident in the clearer aggregation observed in UP-2. Based on the results of the study, it was found that the morphology and ability of peptides are influenced by the balance of hydrophilic and hydrophobic amino acids arranged in the peptide chain.(31) Peptides with many hydrophobic residues tend to form β -sheet structures, which lead to the formation of aggregates or fibrils. The amyloid fibril structure is not clearly observable because the microscope

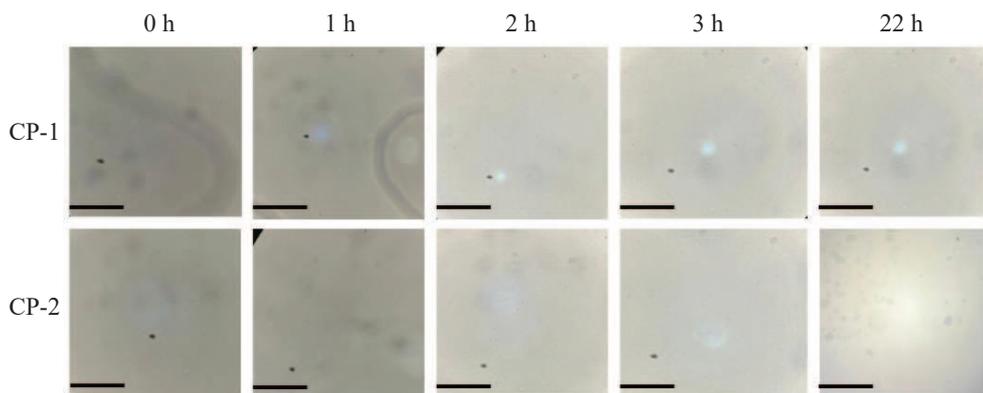


Figure 4. Observation of sea cucumber peptide aggregation using Congo Red staining under a light microscope, monitored from 0 h to 22 h. Peptide aggregation was not detected at this magnification. Black bar: 20 μm.

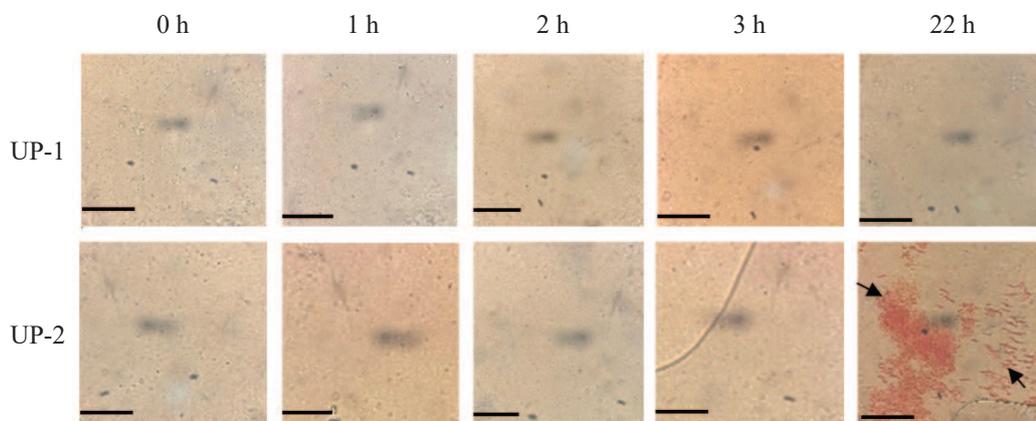


Figure 5. Observation of sea urchin peptide aggregation using Congo Red staining under a light microscope, monitored from 0 h to 22 h. The black arrow in UP-2 (22 h) indicates peptide aggregation. Black bar: 20 μ m.

is limited to a resolution of 200 nm, while the CR-absorbed peptide aggregates are too small, too dispersed, or too amorphous to be clearly seen directly as distinct structures. Therefore, a Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM) microscope is needed to show changes in the size and morphology of the aggregates over time, from monomers to plaques, in order to observe aggregates with micrometer sizes.(32)

To substantiate the computational findings, *in vitro* assays can be employed for the most promising peptide candidates. The Ellman method can be utilized to verify their AChE inhibitory activity, while the Congo Red assay can evaluate peptide stability and aggregation by detecting β -sheet structures associated with amyloid formation. These experimental assessments will furnish essential evidence supporting the computational predictions and enhance the potential of Echinozoa-derived peptides as stable and effective AChE inhibitors for Alzheimer's therapy.

The results of this study confirm that Echinozoa peptides have the potential as AChE inhibitors with distinct characteristics of inhibition, stability, and aggregation. This provides insight into the relationship between amino acid composition and the biological functions as well as the physical properties of peptides. The limitation of this study lies in the fact that it is still at the *in vitro* testing stage as an initial step in designing peptides that can meet the requirements of biomolecules to cross the BBB. Therefore, further investigations are needed, including peptide fragmentation, toxicity analysis, and more detailed enzyme kinetics assays. Future *in vivo* investigations are also advised to confirm the inhibitory efficacy and neuroprotective properties of the peptides, thereby reinforcing their potential as therapeutic agents for Alzheimer's disease. Additionally, molecular dynamics simulations should be utilized to evaluate the dynamic stability of peptide–AChE complexes

under physiological conditions and to elucidate key residue interactions that influence binding affinity and overall inhibitory performance.

Conclusion

Two peptide candidates from sea cucumbers (SWIGLK) and sea urchins (KTKDLK) show the most significant activity in inhibiting the AChE enzyme through binding to the PAS side of the enzyme compared to other candidates. Stability testing shows that both peptides do not undergo aggregation, remain stable in PBS, and do not interact with Congo Red dye. Hence, this initial identification provides an important basis for selecting the most active peptide candidates that have the potential to be developed as Alzheimer's therapies.

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Authors Contribution

LA and VUA performed the experiments, analyzed data and writing the manuscript draft. TRN designed, supervised the research and revise the manuscript. MDL provided input on experimental design and manuscript writing. All authors discussed the results and approved the final version of the manuscript.

Conflict of Interest

The authors declare no conflicts of interest or competing interests related to the content of this manuscript.

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