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Head-to-Tail Cyclization of α-Conotoxin TxID Leads to Enhanced Stability in Serum

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Highlights

- This article focuses on the importance of peptide backbone cyclization.
- Two-stage selective oxidation was used to form the disulfide bridges of α-conotoxin TxID.
- The serum stability of the peptide was enhanced after head-to-tail cyclization.

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Abstract

Peptide biomolecules have important bioactivities and hence their use in drug design and development studies has increased in recent years. Conotoxins are natural peptides that obtained from cone snail venoms and have the potential to be used for chronic pain treatment, Parkinson's disease, schizophrenia, obesity and cancer due to their effects on the nervous system. However, their use as medicines has been limited as they can be easily broken down by many proteolytic enzymes in the body. Several modification methods are used to overcome these disadvantages. Cyclization of the peptide backbone is one such method and has been used to stabilize various linear peptides. In this study, the N- and C-termini of α-conotoxin TxID with two disulfide bridges were joined using a six amino acid long GGAAGG linker peptide chain to cyclize the peptide backbone and the serum stability of the cyclized peptide was examined. The cyclic TxID peptide remained intact about 50% in human serum after 24 hours.

1. INTRODUCTION

Peptides are biopolymers formed by connecting the N- and C-termini of amino acids to each other by a peptide bond. Since they have many important bioactivities, their use as medicines in the treatment of diseases has increased [1]. For example, Captopril, obtained from the secretions of poisonous snakes, is used in the treatment of hypertension and Eptifibatide is used in the treatment of acute coronary syndrome and unstable angina [2,3]. Likewise, cone snail venom peptides are bioactive molecules and are used in many drug research and development studies [4].

Cone snails are marine mollusks and have bioactive peptides called conopeptides in their venoms to catch their prey and/or defend themselves. Conotoxins are disulfide-rich conopeptides and have therapeutic potential as they are potent antagonists or blockers of many ion channels or receptors [5]. These peptides are of great interest as neuropharmacological tools [6]. For example, for the chronic pain treatment, a *Conus magus* cone snail venom peptide named ω-conotoxin MVIIA is the first synthetic cone snail peptide (Ziconotide, Prialt) approved by the Food and Drug Administration (FDA) [7]. This peptide can selectively bind to N-type voltage-gated calcium ion channels and can be used by patients at the same dose since it is not addictive like morphine. However, Ziconotide is administered by intrathecal injection into the spinal fluid, which is a method that patients do not prefer. Although conotoxins have disulfide bridges, they are not very resistant to proteolytic degradation when taken into the body because they are in linear peptide form (open N- and C-terminus). The utilization of peptides as medicines is limited as they are easily degraded by proteases. For this reason, there are much research that have focused on making these valuable molecules more stable. To eliminate the disadvantages of peptides; grafting [8], reversing the sequence

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(retro-inverso) [9], using diselenide [10], dicarba [11] and triazole bridges [12] instead of disulfide bridges, inversion of stereochemistry of a residue, glycosylation [13] and lipidation [14], capping the two peptide termini or cyclizing the peptide backbone from these ends can be used [15].

A new peptide bond is used to join the N- and C-termini of the peptide to make it a head-to-tail cyclic peptide. Depending on the conformational rigidity, these cyclic peptides can show better bioavailability and therefore better bioactivity compared to linear peptides [16]. The rigidity of cyclic peptides reduces disorder and hence allowing them to bind selectively to their targets such as receptors. Another benefit of the cyclic structure is that these peptides can be more resistant to exopeptidases because of the lack of both termini. For example, Cyclosporine A, isolated from a soil fungus called *Tolypocladium inflatum*, is a best known example of cyclic peptides [17]. Other examples are the AS-48 peptide that has antimicrobial activity [18], the plant-derived SFTI-1 peptide is a trypsin inhibitor peptide [19], and another plant-derived peptide kalata-B1 has insecticidal and anti-HIV activities [20]. The resistance of these cyclic peptides to heat and proteolytic enzymes has led to the idea that linear peptides can also become more stable by cyclization [21].

The three-dimensional (3D) structures of the peptides must not be disturbed as they are important for the bioactivity when the peptides are cyclized to enhance their stabilities [22]. A linker chain consisting of amino acids can be used to maintain the tertiary structure. The length of the chain should be chosen according to the distance between the two ends in the three-dimensional structure of the folded linear peptide, and the amino acids used should provide conformational flexibility. Glycine and alanine amino acids are the preferred amino acids because they are small and can minimize the change in the three-dimensional structure of the peptide [23,24].

In Table 1, examples for cyclized conotoxins are shown. Cyclic forms of the α-conotoxin AuIB peptide having linker chains consisting of three to seven residues (cAuIB-3, -4, -5, -6 and -7) have been reported to be more stable to chymotrypsin than its linear form [25]. Vc1.1 is another α-conotoxin and a potent and selective inhibitor of the α9α10 nicotinic acetylcholine receptors (nAChRs). Cyclic Vc1.1 (cVc1.1), synthesized using a six amino acid linker chain, had significantly increased stability in human serum and inhibited rat α9α10 nAChRs with a higher selectivity compared to linear Vc1.1 [26]. α-conotoxin MII, which has the potential to be used in Parkinson's disease, is a potent inhibitor that inhibits the α6 subtype of nAChRs. The stability of the MII peptide was increased in serum by cyclization using a linker chain of six and seven amino acids (cMII-6 and cMII-7) compared to its linear form [23]. TxIB [27] and MVIIA [28,29] are other examples for cyclized conotoxin peptides.

Table 1. *Cyclized conotoxin examples reported in literature*

Conotoxin	Conus Type	Sequence	Reference
AuIB	*Conus aulicus*	GCCSYPPCFATNPDC*	[25]
Vc1.1	*Conus victoriae*	GCCSDPRCNYDHPEIC*	[26]
MII	*Conus magus*	GCCSNPVCHLEHSNLC*	[23]
TxIB	*Conus textile*	GCCSDPPCRNKHPDLC*	[27]
MVIIA	*Conus magus*	GKGAKCSRLMYDCCTGSCRSGKC*	[28,29]

* Amidated C-terminus

α-conotoxins isolated from cone snails can inhibit nAChRs [30]. The α-conotoxin TxID is a 15 amino acid long peptide isolated from the *Conus textile* and very valuable for elucidating the diverse roles of α3β4 nAChRs [31]. It is a member of the α-4/6 conotoxin subfamily and blocks the subtype of α3β4 nAChRs selectively. To reveal the amino acids that are responsible for peptide bioactivity, an alanine scan was performed except the four Cys residues of TxID. The peptide lost its activity at the α3β4 nAChRs when the 5^{th}, 6^{th}, 7^{th}, 11^{th}, and 13^{th} residues were replaced with alanine. The activity of the [G1A] TxID analog was reduced by approximately 19 times compared to the native TxID peptide. [S4A] TxID, [S9A] TxID, [S12A] TxID, and [I14A] TxID analogues almost completely preserved their activities [32,33]. Another study is the pegylation of the TxID peptide. An attempt was made to enhance the stability of the peptide by attaching a DSPE-PEG (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[hydroxyl succinimidyl (polyethylene

glycol)-2000]) to the N-terminus of the TxID peptide. It has been reported that the stability of the peptide had increased in both simulated gastric and intestinal fluids and also in serum, relative to the linear TxID [34]. In a study conducted in 2021, the peptide backbone of [S9A]TxID analogue was cyclized using linker sequences with different lengths. The c[S9A]TxID-6 analogue that has the linker chain with six amino acids was reported to be more stable towards proteinase K enzyme than the linear [S9A]TxID. However, the activities of all synthesized cyclic peptide analogues (c[S9A]TxID-2, -3, -4, -5, -6, -7) against nAChR subtypes decreased [35].

By inhibiting the α3β4 nAChR subtype, α-conotoxin TxID can be used as a potential pain reliever in the treatment of neuropathic pain, as well as a potential drug in the treatment of addiction. However, like most peptides, the poor stability of the α-conotoxin TxID limits its use as a medicine. In this study, peptide backbone cyclization of α-conotoxin TxID and stability of the cyclized peptide were studied. As shown in Figure 1, the N- and C-termini of the peptide were joined using a six amino acid long GGAAGG linker peptide chain.

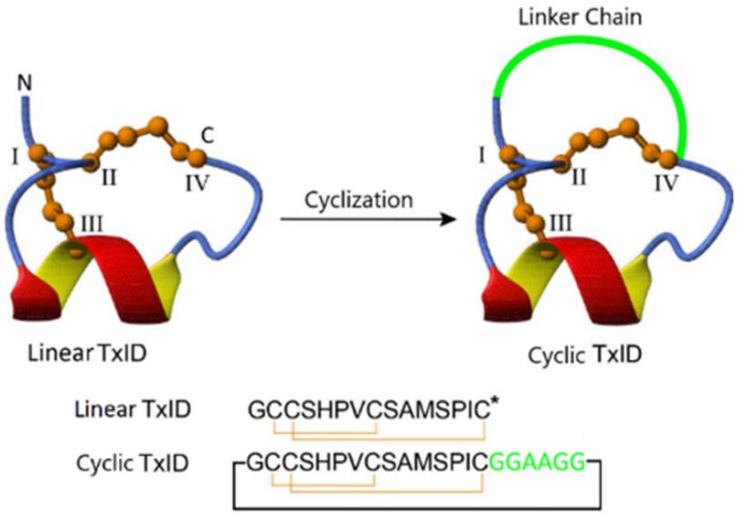

Figure 1. *Cyclization of the linear TxID peptide. The 3D structure of the TxID was shown at the top and prepared with MOLMOL* [36]*. The two disulfide bridges (Cys2-Cys8, Cys3-Cys15) are shown as orange ball-and-stick format and the linker chain of the cyclic TxID was shown in green. The amino acid sequences of both linear and cyclic TxID were shown at the bottom. The disulfide bridges are shown as orange lines and the linker chain GGAAGG is shown in green. The cyclic peptide backbone is shown as black line. The "*" indicates the native linear TxID is amidated at C-terminus*

2. MATERIAL METHODS

2.1. Peptide Synthesis and Cyclization

9-fluoromethyloxycarbonyl (Fmoc) chemistry was used for the synthesis of peptides as illustrated in Figure 2 [37]. Briefly, each amino acid containing Fmoc protecting groups was individually coupled on 2-chlorotrityl chloride (2-CTC) resin, starting from the carboxyl end of the peptide. Fmoc protected amino acids such as Cys(Trt), Cys(Acm), His(Trt) and Ser(tBu) with side chain protecting groups were used to prevent unwanted reactions through the side chains during the synthesis. By using *S*-trityl (Trt) protecting group for Cys2 and Cys8; and *S*-acetamidomethyl (Acm) protecting group for Cys3 and Cys15 side chains, disulfide bridges were ensured to be as in the native peptide (Cys2-Cys8, Cys3-Cys15) during oxidation reactions. Each amino acid was activated and coupled in a solution containing 0.5 M HBTU (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) prepared in *N*,*N*-dimethylformamide (DMF) and concentrated *N*,*N*-diisopropylethylamine (DIPEA) for 2 hours. The Fmoc protecting group of the coupled amino acid was removed using 20% piperidine solution prepared in DMF, and the coupling reaction of the next amino acid was started. After the completion of the peptide chain, the resin was washed and dried with DMF and then with dichloromethane (DCM), and the synthesized peptides were cleaved off from the resin with DCM containing 1% trifluoroacetic acid (TFA). For this purpose, the resin was

incubated in 10 mL of 1% TFA/DCM (v/v) for 10 minutes and filtered. This process was repeated 10 times to ensure that all peptide chains were cleaved off from the resin. DCM was removed by a rotary evaporator and the peptide was lyophilized. The molecular weight of the peptide with side chain protecting groups was analyzed by a Agilent 6530 Quadruple Time of Flight (q-TOF) liquid chromatography mass spectrometry (LC-MS) with electrospray ionization (ESI).

The backbone cyclization of the peptide was achieved as in the literature [37,38]. Firstly, the lyophilized peptide was dissolved in a round-bottom flask with DMF (2 mM) and the N- and C-terminus of the peptide were linked by a new peptide bond for three hours in presence of HBTU (5 mM) and DIPEA (10 mM) as a standard amino acid coupling. After the cyclization reaction, the peptide was stirred in a 50 mL of TFA solution containing 2% TIPS (triisopropylsilane) and 2% H_2O for three hours to remove the protecting groups on the peptide chain except Acm groups on Cys3 and Cys15, and then the TFA was removed by evaporator. The peptide was extracted three times with H_2O/diethyl ether and the aqueous phase was lyophilized. Then, the lyophilized peptide was purified by reversed phase high pressure liquid chromatography (RP-HPLC) (Shimadzu LC-20AT with a UV detector capable of measuring absorbance at wavelengths of 215 nm and 280 nm). GL Sciences preparative C18 column (Inertsil 20x250 mm, WP300, 5 µm, maximum flow rate: 8 mL/min) was used for purification. In the purification process, solution A (H_2O/0.05% TFA (v/v)) and solution B (90% CH_3CN/10% H_2O/0.045% TFA (v/v/v)) were used as mobile phases with a 0.5% gradient method. The method was completed in 140 minutes at a flow rate of 8 mL/min. Mass spectrometry was used to find the fractions of the desired peptide and the fractions containing reduced cyclic TxID were combined and lyophilized for oxidation reactions.

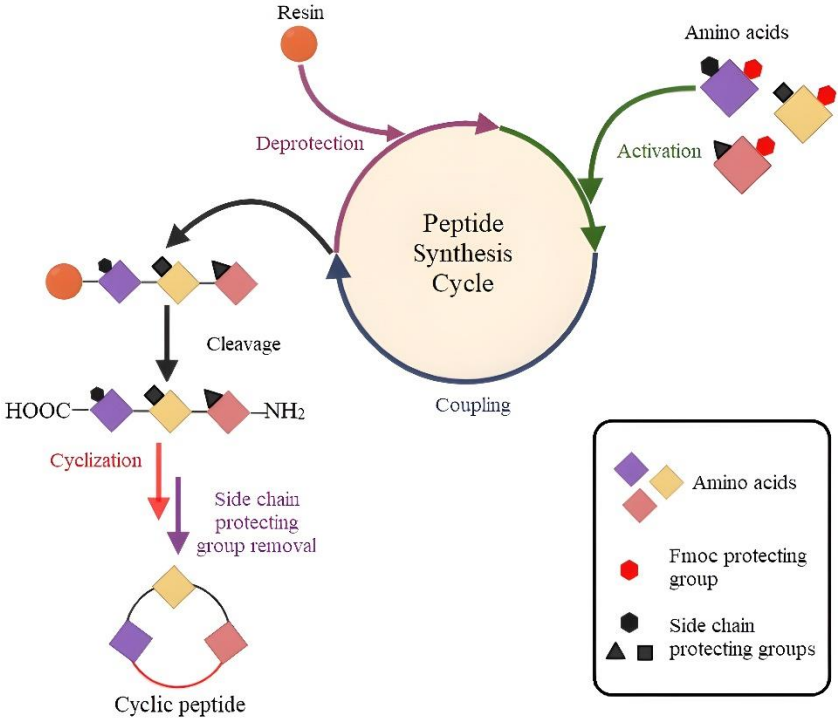

Figure 2. *Schematic representation of peptide synthesis and cyclization*

2.2. Folding and Purification

A two-step selective oxidation reaction was carried out for folding (disulfide bond formation) of the purified cyclic peptide. In the first step, oxidation of Cys amino acids (Cys2-Cys8) with S-trityl protecting groups was carried out in 80 mL of a 0.1 M ammonium bicarbonate (NH_4HCO_3) buffer solution (pH 8.3) with stirring for 24 h. GL Sciences preparative C18 column (Inertsil 20x250 mm, WP300, 5 µm, maximum flow rate: 8 mL/min) was used to purify the peptide with RP-HPLC. In the purification process, solution A and solution B were used as mobile phases with a 0.5% gradient method. The method was completed in 140 minutes. After purification, the peptide was lyophilized. To form the second disulfide bridge (Cys3-Cys15),

the lyophilized peptide was dissolved in a 10 mL of 1:1 mixture of solution A:solution B (v/v). 1 mL of iodine solution was added and stirred for 45-60 min in the dark and the experiment was stopped with ascorbic acid (ascorbic acid was added until the solution turned from brown color to transparent). Thus, removal of the Acm groups and formation of the second disulfide bridge were achieved. The mixture was again purified by GL Sciences preparative C18 column (Inertsil 10x250 mm, WP300, 5 μm, maximum flow rate: 3 mL/min) with RP-HPLC with a 0.5% gradient method for 140 min. The purity of the fractions with the correct molecular weight was analyzed with analytical RP-HPLC. GL Sciences C18 column (Inertsil 2.1x150 mm, WP300, 5 μm, flow rate: 0.3 mL/min) was used for analytical RP-HPLC analyses. Fractions with the same molecular weight and same retention times of the cyclic TxID were combined and lyophilized for the serum stability assay.

2.3. Serum Stability

100% human blood serum (Sigma Aldrich) was used in serum stability experiments [24]. It was centrifuged at 15000 rpm for 10 minutes to separate the lipid components in the serum and the supernatant was incubated at 37 °C for 15 minutes before analysis. Each peptide was added to three eppendorf tubes containing serum at a final concentration of 20 μM. 40 μL aliquots were taken from each tube at 0 (at the time of first addition), 1, 2, 3, 6, 10, 16 and 24 hour time intervals and 6 M of urea solution (40 μL) was added into each tube. The tubes were kept at 4 °C for 10 minutes, and then 40 μL of 20% trichloroacetic acid (TCA) solution was added, allowing the enzymes in the mixture to precipitate. The samples were incubated at 4 °C for 10 minutes and centrifuged at 15000 rpm, and the supernatant (100 μL aliquot) was analyzed by analytical RP-HPLC at a wavelength of 215 nm. For the control experiments, the same procedures were performed using PBS instead of serum. The intact peptide amount was calculated after integration of the peak areas in the chromatogram.

3. RESULTS AND DISCUSSION

3.1. Peptide Synthesis and Cyclization

A six amino acid long GGAAGG sequence was used as a linker chain to maintain the native TxID peptide fold after cyclization. By choosing glycine and alanine amino acids, the side groups of the linker chain amino acids are kept small and thus, it was aimed to prevent the formation of undesired new interactions.

Figure 3 below shows the RP-HPLC chromatogram and the LC-MS spectrum of the peptide after cyclization and removal of the protecting groups except two Acm groups. The cyclic peptide eluted at 62.1 minutes and its molecular weight is 1988.7906 Da (observed mass to charge ratios (m/z) are; $[M+H]^{+}$= 1989.7906, $[M+2H]^{2+}$= 995.3950, and $[M+3H]^{3+}$= 663.9343). According to the LC-MS results, the cyclization of the peptide was successful.

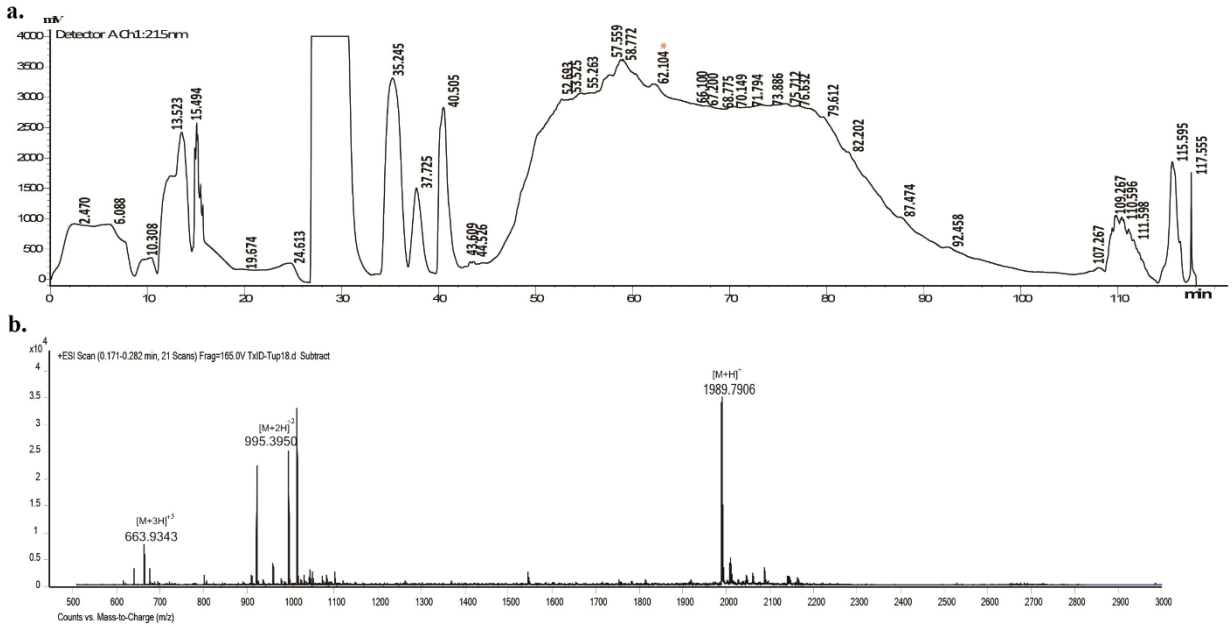

Figure 3. *a) RP-HPLC chromatogram and b) LC-MS spectrum of cyclized TxID peptide after removal of the protective side groups*

3.2. Folding and Purification

The first oxidation step of the peptide after cyclization was carried out in NH_4HCO_3 buffer solution and the first disulfide bridge was formed between Cys2-Cys8 as shown in Figure 4.

Figure 4. *The disulfide bridge is shown as orange line and the linker chain amino acids are shown in green. The Acm side chain protecting groups of the other two cysteine residues are shown above the sequence and the cyclic peptide backbone is shown as black line*

As shown in Figure 5a below, the cyclic TxID peptide with a single disulfide bridge eluted at 67.9 minutes in RP-HPLC. The m/z ratios of the peptide with a molecular weight of 1986.7695 Da according to LC-MS analysis is shown in Figure 5b (observed mass to charge ratios (m/z) are; $[M+H]^+$=1987.7776, $[M+2H]^{2+}$= 994.3919 and $[M+3H]^{3+}$= 662.9290 confirming the molecular weight of the peptide).

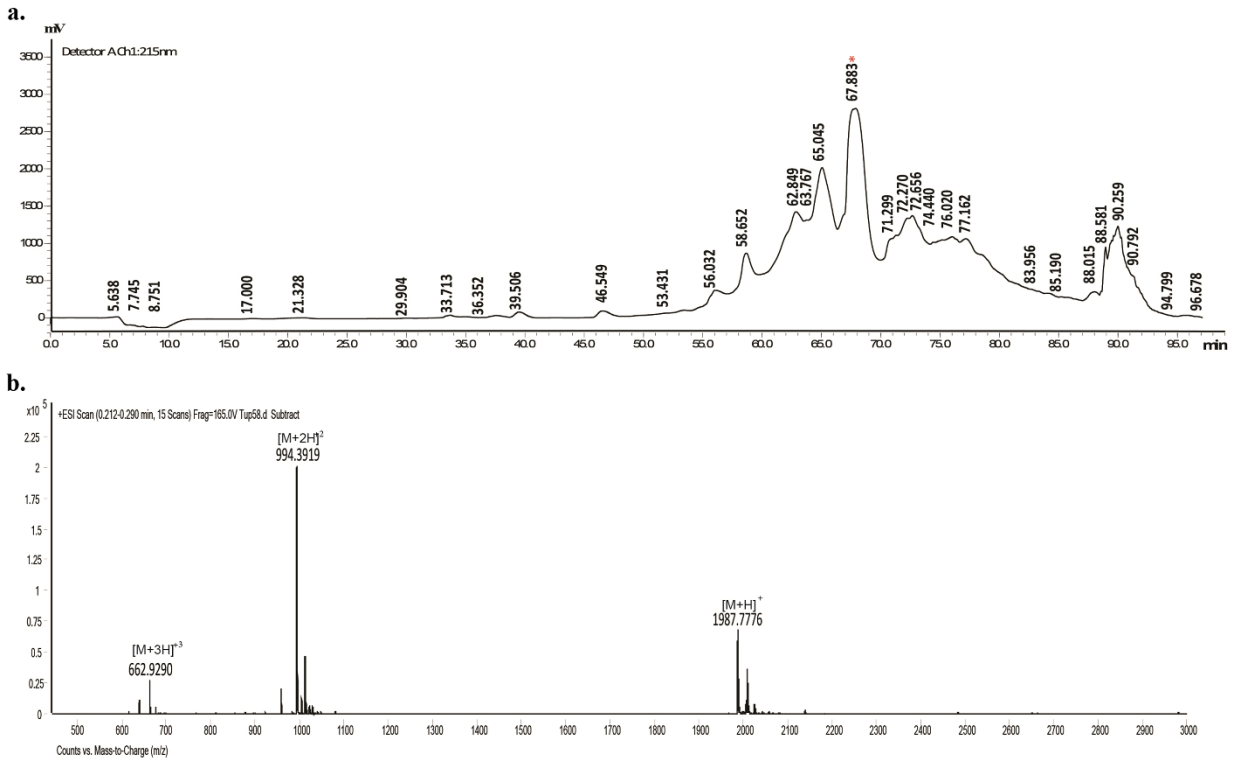

Figure 5. *Purification of the cyclic TxID peptide with a single disulfide bridge by RP-HPLC after first oxidation and determination of molecular weight by LC-MS; a) RP-HPLC chromatogram of the peptide b) LC-MS spectrum of the peptide*

In general, disulfide bridges of α-conotoxins are formed by a two-step oxidation reaction to get the native disulfide bridge conformations. In 2014, the α-conotoxin TxIA peptide was synthesized and folded using both one-step and two-step oxidation strategies. Trt and Acm protecting groups were used for cysteines in the two-step oxidation. It was reported that it is more convenient to perform peptide folding using two-step oxidation strategy [39]. Trt, Acm, tert-butyl (tBu) and methylbenzyl (Mebz) protecting groups are generally used in two-step oxidation in conotoxins [40]. For example, for the synthesis of α-conotoxin BuIA, oxidation was carried out using Mebz and Acm groups for cysteines [41]. Trt, Acm, Mebz protective groups were also used for folding of the α-conotoxin IMI peptide [42].

For the second oxidation of cyclic TxID, the two Acm protecting groups on Cys thiols were removed with iodide solution and a second disulfide bridge was formed as in Figure 6.

Figure 6. *Two disulfide bonds are shown as orange lines and the linker chain amino acids are shown in green. The cyclic peptide backbone is shown as black line*

Figure 7 below shows the RP-HPLC chromatogram, LC-MS spectrum and analytical RP-HPLC chromatogram of the cyclic peptide after formation of the second disulfide bridge. 1.8 mg of fully oxidized cyclic TxID peptide with a purity of more than 95% was obtained. The peptide retention time is 17.85 minutes.

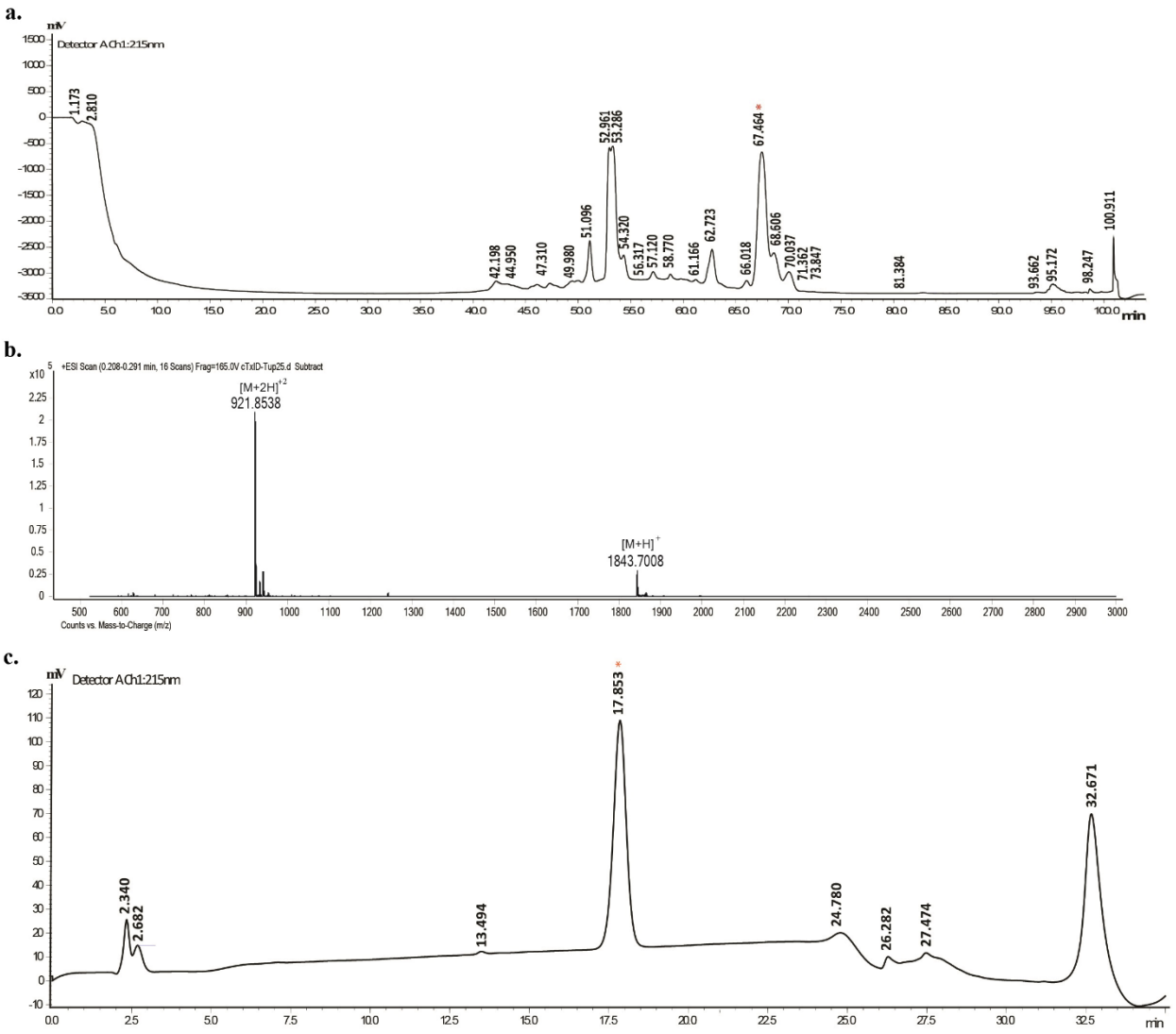

Figure 7. *a) RP-HPLC chromatogram b) LC-MS spectrum and c) Analytical RP-HPLC chromatogram of the cyclic TxID after formation of the second disulfide bridge*

3.3. Serum Stability

The serum stability of the cyclic TxID was tested in 100% human serum. Analytical RP-HPLC was used to determine the amount of the intact peptide after each time interval. The peptide remained 98% intact in serum after the first six hours of incubation as shown in Figure 8. It remained 73.6% intact at the end of 12 hours of incubation and 50% at the end of 24 hours.

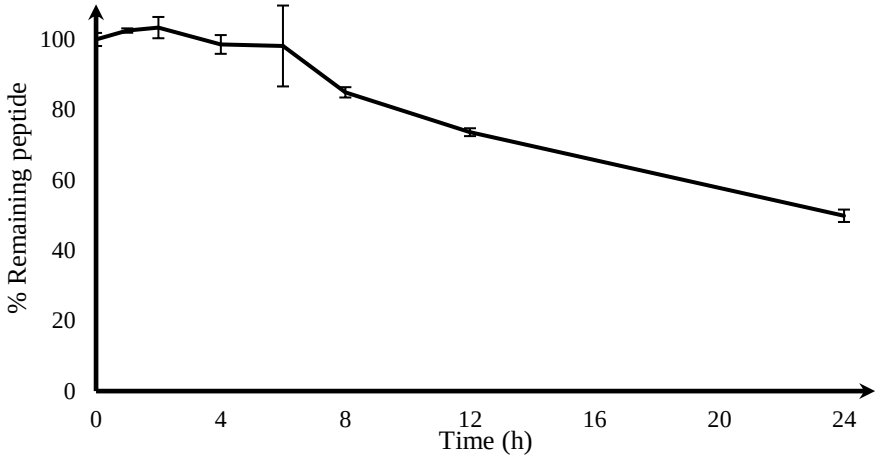

Figure 8. *Stability of cyclic TxID in human serum over 24 hours.*

In a study conducted in 2019 by Weinan Zhao et al., it was reported that the linear TxID peptide could remain in 100% human serum for less than 30% after 24 hours of incubation [34]. The cyclic TxID peptide we synthesized in this study has become more stable in human serum compared to the linear TxID peptide, as shown in Figure 8. In a study conducted in 2020 by Xincan Li et al, the cyclic analogues of the α-conotoxin TxIB peptide formed using a linker of five and seven amino acids remained more than 80% intact after 24 hours in human serum [27]. Another α-conotoxin peptide, RgIA, remained intact in human serum for about 10% in 8 hours, but the cyclic analogs of the peptide with linker chains of six and seven amino acids remained 30% intact, which was the best result among other cyclic analogs of this peptide [43]. It has been reported that the linear Vc1.1 remained less than 80% stable in human serum at 24 hours, and this rate was greater than 80% after cyclization [44]. According to the results in this study, the cyclic TxID peptide remained more than 80% intact in 8 hours and 50% intact after 24 hours in serum.

4. CONCLUSION

nAChRs are ligand-gated ion channels commonly found in both central and peripheral nervous systems that mediate rapid synaptic transmission. The aim of this study was to stabilize the nAChR targeting α-conotoxin TxID peptide by cyclization method. The N- and C-termini of the peptide were joined using a six amino acid long GGAAGG linker peptide chain. The TxID peptide was cyclized and its stability in serum was examined. In conclusion, the α-conotoxin TxID peptide, which can be used in the treatment of various diseases, was converted into a more stable peptide in human serum by head-to-tail cyclization compared to linear TxID. The synthesized cyclic peptide with higher serum stability can reach its target and show its therapeutic effect on nAChRs.

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CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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