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Antioxidant and angiotensin-converting enzyme (ACE) inhibitory activity of thymosin alpha-1 (Thα1) peptide

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ABSTRACT

In this research, the antioxidant property of thymosin alpha-1 (Thα1) peptide was investigated through various antioxidant methods. Thα1 showed 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (IC_{50} = 20 μM) and its 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) scavenging reached 45.33% at 80 μM (IC_{50} = 85 μM). In addition, hydroxyl and superoxide radical scavenging of Thα1 peptide exhibited a concentration-depended manner. The IC_{50} values of hydroxyl and superoxide radical scavenging were estimated to be 82 μM and 20 μM, respectively. The effect of Thα1 on eliminating superoxide radicals was higher (62.23%) than other antioxidant assays. Moreover, the antioxidant activity of Thα1 peptide was evaluated by measuring cellular reactive oxygen species (ROS). Results indicated that Thα1 decreased the generation of ROS level in 1321 N1 human neural asterocytoma cells. The inhibitory effect of Thα1 on angiotensin-converting enzyme (ACE) was determined. The kinetic parameters (K_m and V_{max}) and the inhibition pattern were examined. Based on the Lineweaver-Burk plot, Thα1 displayed a mixed inhibition pattern. The IC_{50} and K_i values of Thα1 were 0.8 μM and 3.33 μM, respectively. Molecular modeling suggested that Thα1 binds to ACE-domains with higher affinity binding to *N*-domain with the binding energy of −22.87 kcal/mol. Molecular docking indicated that Thα1 interacted with ACE enzyme (N- and C-domains) due to electrostatic, hydrophobic, and hydrogen forces. Our findings suggested that Thα1 possess a multifunctional peptide with dual antioxidant and ACE-inhibitory properties. Further researches are needed to investigate the antioxidant and anti-hypertensive effect of Thα1 both *in vitro* and *in vivo*.

1. Introduction

In recent years, natural peptides have been the subject of intense research. These peptides are short-chained polypeptides up to 50 amino acids, which have critical roles in various physiological processes [1]. They naturally occur in all living organisms [2] and display very specific biological activities, which are primarily based on their sequences and ultimately dependent on their structures. They are involved in different biological processes as agonists, antagonists, mediators, hormones, cofactors, and activators [1]. Nowadays, several natural peptides have been identified with antimicrobial [3], antioxidant [4], anticancer [5] properties and some of them have shown a key role as an inhibitor of angiotensin-converting enzyme (ACE)[6]. Hypertension is considered as the main causes of death worldwide [7]. It is estimated that approximately 1 billion of the world's population is infected by high blood pressure [8]. High blood pressure is associated with increased heart failure, coronary artery disease, and ischemic heart diseases and is elevated by factors as increasing the intake of salt, body weight, smoking, and some genetic factors [9,10]. Renin-angiotensin system (RAS) is a hormone system that plays a vital role in controlling blood pressure and fluid balance [11]. ACE as an essential enzyme in the RAS system causes a rise in blood pressure by catalyzing the reaction of the inactive decapeptide angiotensin-1 to the active vasoconstrictor angiotensin-2 [6]. Therefore, reducing the concentration of angiotensin-2 by suppressing ACE in the body is of great importance. Small inhibitor molecules of ACE are extremely used as therapeutic drugs for the treatment of cardiovascular events [12,13]. Several ACE inhibitors are available including benazepril, captopril, lisinopril, Ramipril, and trandolapril that are used as synthetic drugs for the treatment of high blood pressure [12,14]. Antioxidant activity is considered as another basic function of natural peptides. Reactive oxygen species (ROS) are generated through inflammatory, endothelial, and immune cells due to different cellular pathways [15]. The increase in ROS causes damage to biomolecules such as DNA and protein through a process

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known as oxidative stress [16]. As a result, these conditions are associated with a wide range of diseases including cardiovascular events [17], diabetes [18], cancer, and inflammation [19]. Therefore, it is of great importance to identify natural peptides that can protect against the destructive effects of oxidative stress. In this regard, different natural peptides with antioxidant capacity have been reported. It seems that these peptides are safer and have lower side effects in comparison with synthetic peptides [20]. Thymosin alpha-1(Thα1) is a 28 amino acid peptide (SDAAVDTSSEITTKDLKEKKEVVEEAEN) purified from calf thymuses [21]. The Thα1 peptide is widely found in the thymus, lung, brain, blood, and kidney [22]. Thα1 has been observed to increase the response of T lymphocytes and induce the generation of interleukins and interferons [23,24]. This natural peptide has demonstrated beneficial antitumor activity both *in vitro* and *in vivo* [21]. Lowry et al. indicated that Thα1 has the ability to induce the proliferation of mouse splenic lymphocytes and enhance apoptosis in the tumor cells [25]. Due to biological properties of Thα1, it has gained great interest in the recent years [21] and is used as an effective peptide for the treatment of some diseases including hepatitis B and hepatitis C, several types of cancer (e.g., melanoma and HCC), and immunodeficiency diseases (e.g., aging and autoimmune disease) [21,26]. In this study, the inhibitory effect of Thα1 on the activity of ACE and its inhibition mechanism is investigated. Next, the interaction between ACE-domains and Thα1 was determined using molecular docking. Moreover, the antioxidant properties of Thα1 were evaluated using ABTS, DPPH, superoxide, and hydroxyl radical scavenging assays.

2. Material and methods

2.1. Reagents

Thymosin alpha 1 (Thα1) with purity 95% was chemically synthesized by GL Biochem (Shanghai, China) and used without further purification. 1, 1-diphenyl-2- pycryl-hydrazyl (DPPH), 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonicacid) (ABTS), ferrozine, glutathione reduced (GSH), 1, 10-phenanthroline, angiotensin converting enzyme (ACE) extracted from rabbit lung and N-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly (FAPGG) were obtained from Sigma (St. Louis, MO, USA). In addition, ferrous sulfate, hydrogen peroxide (H_2O_2), ethylenediaminetetraacetic acid (EDTA) and pyrogallol were purchased from Merck Chemicals Co. (Darmstadt, Germany). 2,7- dichlorohydrofluorescein-diacetate (DCFH-DA), dimethyl sulfoxide (DMSO), DMEM (Biosera, East Sussex, UK), Fatal Bovine Serum (FBS) (Gibco, Grand island, NY, USA), penicillin/streptomycin (Biosera, East Sussex, UK), trypsin (Biosera, EastSussex, UK) and phosphate-buffered saline (PBS) were of analytical grade.

2.2. Antioxidant activity of Thα1

2.2.1. DPPH radical scavenging assay

DPPH radical scavenging activity was conducted using the method explained by Göçer and Gülçin [27]. Initially, 400 µl of DPPH solution (0.15 mM in 95% ethanol) was mixed with 100 µl of various concentration of peptide (5, 10, 20, 40 and 80 µM) and the mixture was placed at darkness for 30 min. After incubation, the absorbance of samples was measured at 517 nm. For control, 100 ml of distilled water was used instead of the peptide. DPPH radical scavenging was calculated according to the following equation:

$$\text{DPPH radical scavenging}\,(\%) = [(A_C - A_S/A_C) \times 100]$$

2.2.2. ABTS radical scavenging assay

ABTS radical scavenging was evaluated based on the methodology explained by Tironi and Anon [28]. In brief, ABTS radical solation was prepared by mixing 2 ml of ABTS (7 mM) with 1 ml of potassium persulfate (2.45 mM). The reaction mixture was maintained at a dark place for 12–16 h. Then, the ABTS working solation was diluted with distilled water to set the absorbance to 0.756 at 734 nm. Next, 400 µl of ABTS radical solation was mixed with 100 µl of various concentration of Thα1 and the resulting solution was kept at 37 °C for 1 h. Eventually, the absorbance of the samples was measured at 734 nm. GSH was considered as positive control. ABTS radical scavenging was calculated through the following equation:

$$\text{ABTS radical scavenging}\,(\%): [(A_C - A_S/A_C) \times 100]$$

2.2.3. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging capacity was estimated as explained previously [29]. In summary, 100 µl of ferrous sulfate (5.0 mM) was mixed with 100 µl of various concentration of Thα1 and the mixture was placed at room temperature for 3 min. Then, 140 µl of H_2O_2 (0.01%) and 100 µl of 1,10-phenanthroline (5.0 mM) were added to the pervious mixture. The reaction mixture was incubated at 37 °C for 60 min.

Subsequently, the absorption of the samples was measured at 536 nm and hydroxyl radical scavenging was evaluated using the equation below:

$$\text{Hydroxyl radical scavenging activity}\,(\%): [(A_s - A_c/A_b - A_c) \times 100]$$

2.2.4. Superoxide radical scavenging assay

Superoxide radical scavenging activity was investigated based on the method discussed by Pownall and colleagues [30]. In brief, 80 µl of the Thα1, 80 µl of 50 mM Tris–HCl solution (pH 8.3, 1 mM EDTA) were poured into a 96-well plate. Afterward, 40 µl of pyrogallol (1.5 mM in 10 mM HCl) was added and the mixture was shaken vigorously. A mixture of Tris–HCl buffer (50 mM, pH 8.3) and pyrogallol without peptide was considered as control. GSH was utilized as positive control. Ultimately, the absorption of the samples and the control was determined using an ELISA reader at 420 nm. The following equation was applied to evaluate the percentage of hydroxyl radical scavenging:

$$\text{Superoxide radical scavenging}\,(\%) = [(\text{A control} - \text{A sample})/\text{A control}) \times 100]$$

2.2.5. ROS antioxidant assay

The production of ROS was evaluated via 2,7- dichlorohydrofluorescein- diacetate (DCFH-DA). Nearly, 1×10^5 human neural astrocytoma cells (obtained from Pasteur Institute, Tehran, Iran) were seeded in 24-well plate and maintained at 37 °C for 24 h. Next, the cells were treated with various concentrations (10, 20 and 40 µM) of Thα1 and placed for 24 h. Then, 50 µl of DCFH-DA fluorescence probe was added to each well and incubated at 37 °C for 30 min. The cells were removed from the surface of the plate by adding 200 µl of trypsin/EDTA. The trypsin solution was neutralized by adding 700 µl of the culture medium and then the cells were transferred to 1.5 ml eppendorf. The human neural astrocytoma cells were centrifuged at 4,000 rpm for 5 min. After than the supernatant was discard and 300 µl of PBS was added. Finally, the cells were transferred to flow-cytometric tubes and the fluorescent signals were detected through flow-cytometry.

2.3. ACE inhibition

2.3.1. Evaluation of ACE inhibitory activity

ACE inhibitory activity was evaluated using FAPGG as substrate based on the method explained by Holmquist et al. [31]. One unit of ACE activity is considered as the amount of enzyme which hydrolyzes 1 µmol of FAPGG to FAP and GG per 1 min at room temperature [32]. Initially, to determine the ACE inhibitory effect of Thα1 a reaction mixture containing 175 µl of Tris–HCl buffer (50 mM pH 7.5,1 mM zinc chloride, and 0.3 M NaCl), 50 µl of various concentration of Thα1 and

65 µl FAPGG was prepared and mixed vigorously. Then, 30 µl of ACE solution was added and the absorbance was examined at 334 nm for a period of 3 min. Control sample included 225 µl of Tris–HCl buffer (instead of 50 µl peptide), 65 µl of FAPGG and 30 µl of the enzyme solution. The ACE inhibitory effect of Thα1 was investigated using the absorbance changes (ΔA) of samples and control based on the equation bellow:

$$\text{ACE inhibition}\ (\%) = [1 - \Delta A_{sample}/\Delta A_{control}] \times 100$$

2.3.2. Determination of kinetic parameter of ACE inhibition

ACE inhibitory activity of Thα1 was examined in the presence and absence of Thα1 peptide (0.48 and 0.97 µM), and the results were depicted by Lineweaver–Burk plots. The experiment was carried out at various concentration of FAPGG (0.5, 1, 1.5 and 2 mM) as substrate.

2.3.3. Molecular modeling

The complete three-dimensional structure of the *N*-domain of rabbit ACE was modeled based on method of Tanzadehpanah et al. (2013). In standard docking methods, the receptor is assumed to be rigid whereas the conformers of the ligand are searched [33]. Briefly, the amino acid sequence of ACE was retrieved from http://www.uniprot.org/ (Entry code: P12822). The similarity search using Psi-Blast was then performed against available PDB structures in order to find proteins with resolved structure and high identity as candidate templates. The resultant structure was *N*-domain of human somatic ACE (PDB ID: 2C6N) which was used for modeling of the *N*-domain (35–645 residues) and human testis ACE (PDB ID: 2OC2) was utilized for the modeling of the C-domain (649–1232 residues) of the ACE (rabbit) enzyme. Furthermore, the structure of Thα1 was obtained from protein data bank with PDB ID: 2L9I. ACE-peptide docking study was performed using the Molecular Operating Environment 2008.10 (MOE) software to locate possible peptide binding site on the C- and *N*-domain of ACE. In this work, all water molecules were removed from the protein and peptides file in MOE software. Protonate 3D was utilized in order to add hydrogens, calculate charges and otherwise prepare the receptor, also the Surfaces and Maps was applied to generate visual representation of molecular surface, and to predict the preferred locations of ligand atoms (in the absence of the ligand itself), in order to determine the possible conformation interaction for ACE. The charges of all atoms were assigned using the current Forcefield refinement while the London dG scoring function estimates the free energy of binding of the ligand from a given pose. The docking parameter file for docking procedure was set to 30 runs. Finally, the best docking energy results were assumed as the possible candidates for ligand- protein interaction and then copy molecules from selected entries in the database to MOE by choosing Copy Selected to MOE. Following each docking procedure, the best docking results were submitted to Discovery Studio Visualizer 2.5.5. Moreover, the interaction mode between ACE and peptide was determined by MOE software.

2.3.4. Statistical analysis

Experiments were analyzed through the one-way ANOVA. The results were presented as mean ± SD and values at $p \leq 0.05$ were considered statistically significant.

3. Result and discussion

3.1. Antioxidant assays of Thα1

3.1.1. DPPH radical scavenging activity

DPPH analysis is a common method based on the decrease in DPPH stable radical. This analysis has a strong absorbance at 517 nm (purple color) such that it is widely used for measuring the antioxidant properties of various compounds. Briefly, the reaction of antioxidant compounds with DPPH radical in the presentment of a hydrogen donor

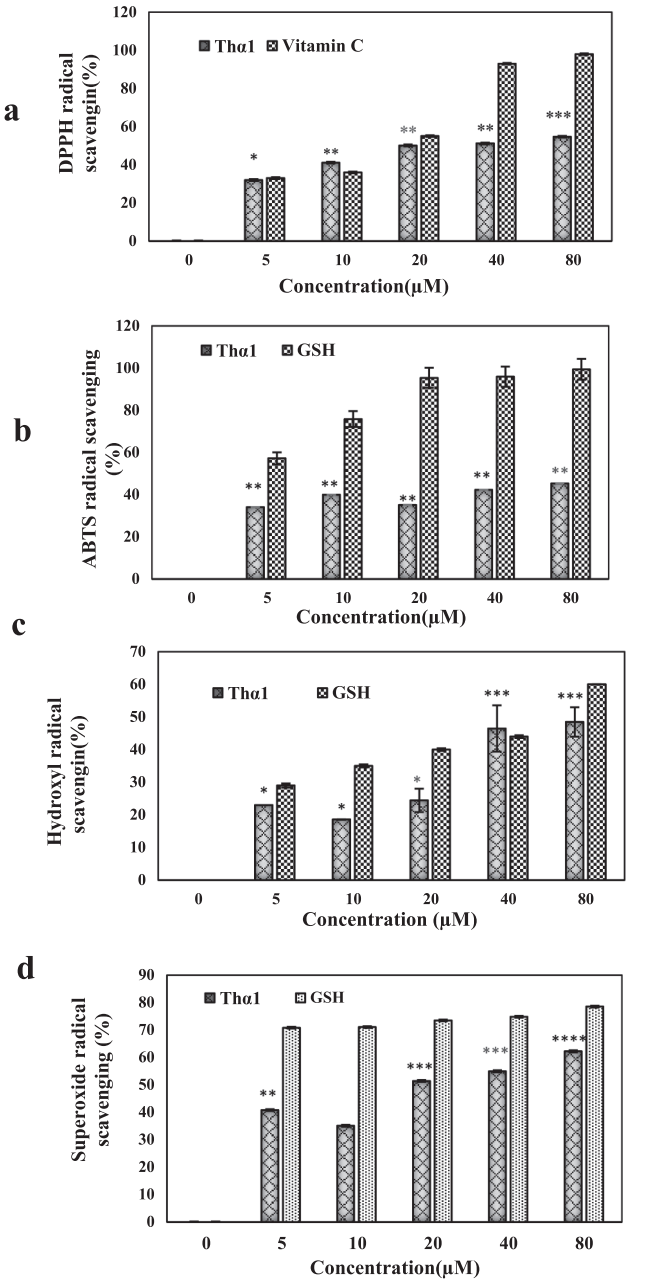


Fig. 1. The antioxidant activity of various concentration of Thα1 in different antioxidant assays. **(a)** DPPH radical scavenging activity of various concentration of Thα1 and vitamin C; **(b)** the effect of Thα1 and GSH on ABTS radical scavenging; **(c)** the effect of Thα1 and GSH on Hydroxyl radical scavenging; **(d)** superoxide scavenging activity of different dosages of Thα1 and GSH.

results in the reduction of DPPH into DPPH-H and thereby reducing the absorption of DPPH (yellow color) [34]. The capacity of Thα1 in DPPH radical scavenging was evaluated using DPPH radical decolorization assay. As shown in Fig. 1a, Thα1 shows a concentration-dependent DPPH scavenging activity that reached 54.68% at 80 µM. The IC_{50} value (50% inhibition dosage) of Thα1 peptide was estimated to be 20 µM. Several studies have emphasized that the composition and the sequence of amino acid affect the antioxidant scavenging activity of the peptides [35-37]. Also, the interactions between amino acids are considered as a major factor for the antioxidant capacity [37]. In general, the presence of hydrophobic amino acids including Gly, Ala, Val, Leu, Ile, Pro, Phe, Met, and Trp indicated a higher antioxidant capacity [38]. For instance, a natural peptide purified from myofibrillar protein

hydro-lysate (FVNQPYLLYSVHMK) demonstrated a strong DPPH antioxidant activity (IC_{50} = 0.268 ± 0.06 mg/ml). It seems that the sequence of the peptide containing hydrophobic amino acids (Leu, Val, and Phe), hydrophilic amino acids (His, Pro and Lys), and aromatic amino acids (Phe and Tyr) stimulates the antioxidant capacity [38]. Another study compared the DPPH scavenging capacity of seven peptides (i.e., LHY, LARL, GGE, GAH, GAWA, PHYL, and GALAAH). The results showed that the first peptide (LHY) exhibited the most DPPH radical-scavenging capacity (63 ± 1.57%) [39]. In the current study, it seems that the presence of hydrophobic amino acids in the sequence of Thα1 including Gly, Ala, Val, Leu, and Ile contributes to DPPH radical scavenging and the antioxidant activity of Thα1 peptide.

3.1.2. ABTS radical scavenging assay

ABTS radical scavenging assay is widely used as a method to investigate the antioxidant activity of various components. In ABTS assay, a green-blue cationic chromophore $ABTS^{\bullet+}$ with strong absorption at 734 nm is generated through oxidation [40]. The ABTS radical scavenging capacity of Thα1 was examined in comparison with GSH (Fig. 1b). As presented in Fig. 1b, Thα1 exhibited a dose-dependent ABTS radical scavenging activity. The scavenger capacity of Thα1 at 20 μM was 35.07% whereas at the same concentration of GSH the radical scavenging activity was 95.38%. Wang et al. isolated two peptides from the hydrolysate of *Sphyrna lewini* muscle with the sequence of WDR and PYFNK. The PYFNK indicated a higher ABTS scavenging activity than WDR peptide and the IC_{50} values were 0.12 mg/ml and 0.34 mg/ml, respectively [41]. Moreover, Wang et al. identified 5 peptides from spotless smoothhound muscle including GAA, GFVG, GIISHR, ELLI, and KFPE. All these peptides showed a high scavenging activity on ABTS and the IC_{50} values were 1.75 mg/ml, 1.30 mg/ml, 0.34 mg/ml, 0.32 mg/ml, and 0.46 mg/ml, respectively [35]. In another work, it was reported that the presence of acidic amino acids in the sequence of peptides including Asp and Glu probably contributes to the ABTS scavenging capacity [37]. Chi et al. purified three peptides from the hydrolysate of croceine croaker muscle (i.e., VLYEE, YLMSR, and MILMR), which exhibited a strong antioxidant capacity (ABTS, DPPH, and superoxide radical scavenging) due to the low molecular weights and the presence of hydrophobic and aromatic amino acids within their sequences [42].

3.1.3. Hydroxyl radical scavenging assay

Hydroxyl radicals are known as the most harmful radicals because of their convenient reaction with biomolecules containing DNA and proteins, which causes serious damages to the cells and tissues [4]. The increase in these radicals is associated with a wide range of diseases [43]. Several antioxidants including vitamin C, butylated hydroxyanisole, and GSH are used in order to eliminate the harmful effects of hydroxyl radicals. The hydroxyl scavenger ability of Thα1 is shown in Fig. 1c. The results show the dose-dependent inhibitory effect of Thα1 on the hydroxyl radicals. Moreover, they showed that at 80 μM of Thα1 the capacity of hydroxyl scavenging is about 48.5%. GSH was used as positive control which exhibited 60% hydroxyl radical scavenging at 80 μM. In one study, Qian et al. isolated a novel peptide (LEELEEELEGCE) from bullfrog skin, which showed a strong hydroxyl scavenging capacity with an EC_{50} value of 12.8 mM. They found that the presence of glutamate in the sequence of this peptide may contribute to antioxidant activity and play a major role as hydrogen donor [44]. Also, Je et al. identified a novel peptide from *Alaska pollack*. The sequence of the peptide was LPHSGY, which exhibited 35% hydroxyl radical-scavenging at 53.6 μM [45].

3.1.4. Superoxide radical scavenging activity

Superoxide radicals are the major elements of ROS. They are generally produced in various cells via the one-electron reduction of the molecular oxygen. These anionic radicals are eliminated in living organisms using the superoxide dismutase. In the present study, the effect of Thα1 on removing $\mathbf{O_2^-}$ radicals was investigated by superoxide radical scavenging activity. As presented in Fig. 1d, Thα1 eliminated the superoxide anions in a concentration-dependent manner. Based on our results, Thα1 at 80 μM showed a 62.23% superoxide radical scavenging activity. GSH was used as a positive control, which exhibited a potent antioxidant capacity (78.52%) at 80 μM. Saidi et al. observed that the nanofiltration fraction (1–4 kDa) from tuna dark muscle by-product containing Phe, Ala, Tyr, Pro, Leu, and His amino acids had a potent capacity to scavenge superoxide radicals [46]. Another study compared the antioxidant activity of three peptides YLMSR, VLYEE, and MILMR isolated from the hydrolysate of croceine croaker muscle. They observed that the YLMSR peptide exhibited the strongest superoxide, ABTS, and DPPH radical scavenging activity such that the EC_{50} values were 0.450 mg/ml, 0.312 mg/ml, and 1.35 mg/ml, respectively [42]. In addition, NADFGLNGLEGLA (IC_{50} 0.864 mg/ml)[47], PYFNK (IC_{50} 0.11 mg/ml)[41], and GVPLT (EC_{50} 2.881 mg/ml) [48] peptides, which were purified from the hydrolysate of giant squid, *Sphyrna lewini* muscle, and *Bluefin leatherjacket* heads, showed the ability to remove superoxide radicals.

3.1.5. ROS measurement

The ROS antioxidant activity was investigated through 2, 7- dichlorohydrofluorescein- diacetate (DCFH-DA) probe. 1321 N1 human neural astrocytoma cells were treated with Thα1 (10, 20 and 40 μM) for 24 h. Results from flow-cytometry analysis indicated that the production of cellular ROS after treating the cells with Thα1 (10, 20 and 40 μM) was lower (23.05%, 19.7% and 7.94%, respectively) compared to control (38.95%) (Fig. 2). Similarly, Qin et al. demonstrated that exposure of HepG2 cancer cells with Thα1 peptide reduced the generation of cellular ROS; however, the level of ROS increased in normal lukomonocyte [49].

3.2. ACE inhibitory activity

3.2.1. ACE inhibition

The ACE inhibitory effect of Thα1 was evaluated using FAPGG as substrate (Fig. 3a). Based on our results, Thα1 exhibited a potent ACE inhibitory activity. The inhibitory effect of Thα1 was estimated from 5 to 84%. The IC_{50} value of Thα1 was examined to be 2.5 μg/ml (0.8 μM). The ACE inhibitory effect of Thα1 was higher than the peptide derived from the fish and shellfish (rich in Asp, Glu, Arg, Pro, Ile and Lys, IC_{50} = 130 μg/ml) [50] and ostrich egg white protein (AFKDEDTEEVPFR, IC_{50} = 127 μg/ml) [51]. Several studies have been conducted on the ACE inhibitory effect of natural peptides. Lee et al. purified a peptide from Brachionus rotundiformis with the sequence of DDTGHDFEDTGEAM. The peptide indicated a potent ACE inhibitory ability

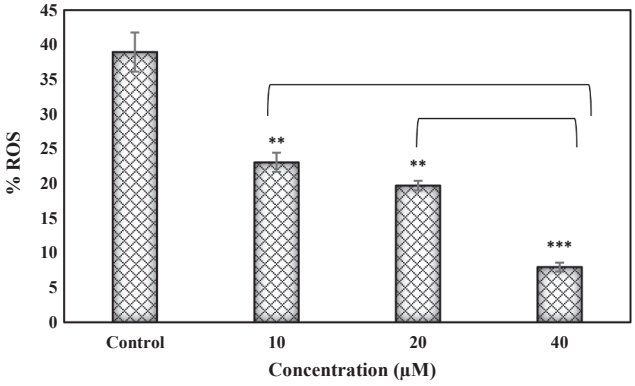
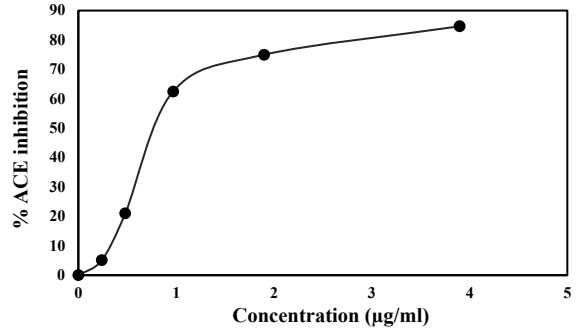


Fig. 2. The percent of reactive oxygen species (ROS) production in human neural astrocytoma cells treated with various concentration of Thα1 after 24 h. Each bar represents the mean ± SD from two experiments. **P < 0.01; ***P < 0.001 compared to control (sample without peptide).



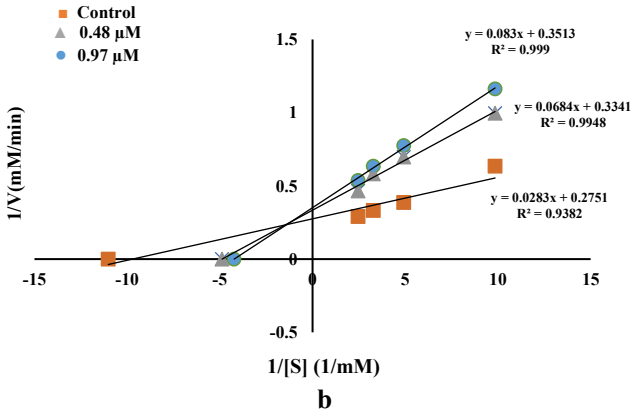


Fig. 3. Determination of ACE inhibitory mechanism. **(a)** The inhibitory effect of Thα1 on ACE enzyme at various concentration of Thα1; **(b)** Lineweaver-Burk plot of Thα1 as inhibitor of ACE at 0.48 µM (▲), 0.97 µM (●) and control (■). The inhibition effect of Thα1 was evaluated in three independent experiments.

with the IC_{50} value of 9.64 µM [52]. Three peptides purified from casein hydrolysate by proteolytic extracted from Lactobacillus (MPFPKYPVEP, EPVLGPVRGPFP, and IGSENSEKTTMP) exhibited ACE inhibitory activity with IC_{50} values of 83, 790, and 733.10 µM, respectively [53]. The amino acid composition of peptides affected ACE inhibitory activity [54]. According to the results of a study [55], Pro at the C-terminus of isolated novel peptides from casein hydrolysate demonstrated the potent ACE inhibitory effect. Similarly, Balti et al. purified two peptides from the muscle of cuttlefish with the sequence of VELYP and AFVGYVLP, which indicated strong ACE inhibition activity (IC_{50} values 5.22 and 18.02 µM, respectively) [12]. Their results showed that the presence of Pro at the C-terminal of these two peptides induced the ACE inhibitory activity [12]. As shown in Table 1, several long-chain peptides with ACE inhibitory activity have been identified from various natural sources. It was reported that the presence of charged amino acids at the C-terminal of peptides has a critical role in the interaction with ACE enzyme [61]. Moreover, the presence of hydrophobic amino acids at the C-terminal and aliphatic amino acids at the *N*-terminal position contributes to the ACE inhibitory action [62]. One study reported that the presence of hydrophobic amino acids in YLYELAR and AFPYYGHHLG isolated from scorpion venom of H. lepturus exhibited a strong ACE inhibition activity [6]. Therefore, our findings suggested that charged and hydrophobic amino acids in Thα1 induce the ACE inhibitory of the Thα1 peptide.

3.2.2. ACE inhibition pattern of Thα1 peptide

The ACE inhibition pattern was evaluated using Lineweaver–Burk plot (Fig. 3b). Our findings demonstrated that Thα1 exhibited a mixed inhibition indicated that Thα1 binds to a site distinct from the precise active site. Mixed inhibitors are bound both ACE-substrate complex and the free enzyme. Results showed that the values of K_m in the absence of Thα1 was 0.1 mM and in the presence of two concentrations, 0.48 µM and 0.97 µM of Thα1 peptide were 0.2 and 0.236 mM, respectively. Moreover, the values of V_{max} in the absence and presence of two different dosages of Thα1 (0.48 µM and 0.97 µM) were 3.7, 3.03 and 2.85 (mM/min), respectively. The inhibitor constant (Ki) for mixed inhibition was investigated using the intercept on the axis of the secondary plot of $1/V_{max}$ against the peptide concentration [I_0].The calculated K_i value for the Thα1 peptide of was 3.3 µM.

3.2.3. Molecular modeling

Molecular docking is used for studying the binding between ligand molecules and macromolecules as an intuitive and effective research method [63]. Therefore, Molecular modeling was performed to confirm the experimental data. Somatic ACE (sACE) is composed of two domains namely C-domain and *N*-domain [64]. The position of the N domain is around a central groove, which is located on the active site (in the Glu-396 position) where the peptides can bind (http://www.uniprot.org/). Also, ACE included three active site pockets (S1, S2, and S1′). S1 contained Glu-384, Tyr-523, and Ala-354 residues while S2 is composed of Tyr-520 His-353, Lys-511, His-513, and Gln-281 residues; however, S1′ included Glu-162 residue [65]. Several of the ACE inhibitors include hydrophobic amino acid in each of the three C-terminal positions, which interact with S1, S1′, and S2 at the active site of ACE [66]. In comparison, some ACE inhibitor peptides are not competitive inhibitors and bind to a site distinct from the active site [67]. In the current study, the best molecular docking outcome, according to the lowest energy level, was obtained. Fig. 4 demonstrates the results of the Thα1 interaction with the C and *N*-domains of ACE. Furthermore, the macroscopic viewers of the interaction between Thα1 and ACE domains are shown in Fig. 4. As shown in Table 2, the binding energy of ACE-Thα1 complex for N- and C-domains were −22.87 and −17.74 kcal/mol, respectively. Our findings suggested that Thα1 has more binding affinity to *N*-domain than C-domain. Furthermore, the molecular

Table 1
The ACE inhibitory activity of some long-chain peptides.

Amino acid Sequence	Source	IC_{50}	Ref
GDLGKTTTVSNWSPPKYKDTP	Tuna	11.28	[56]
GAXGPAGPGGIXGERGLXG	Chicken collagen	45.6	[57]
GLXGSRGERGERGLXG	Chicken collagen	60.8	[57]
GIXGSRGERGPVGPSG	Chicken collagen	43.4	[57]
TKAVEHLDDLPGALSELSDLHAHKLRVDPVNFKLLSHLL	Bovine blood	366	[58]
LDDLPGALSELSDLHAHKLRVDPVNFKLLSHSL	Bovine blood	518.29	[58]
WPEAAELMMEVDP	Tuna	21.6	[59]
DDTGHDFEDTGEAM	Rotifer	9.64	[52]
QTQYTDAPSFSDIPNPIGSENSEKTTMPLW	α_{s1}-casein	346	[60]
DELQDKIHPFATQSLVYPFPGPIPNS	ß- casein	4	[60]
LLYQQPVLGPVRGPFPIIV	ß- casein	21	[60]
YQQPVLGPVRGPFPIIV	ß- casein	101	[60]

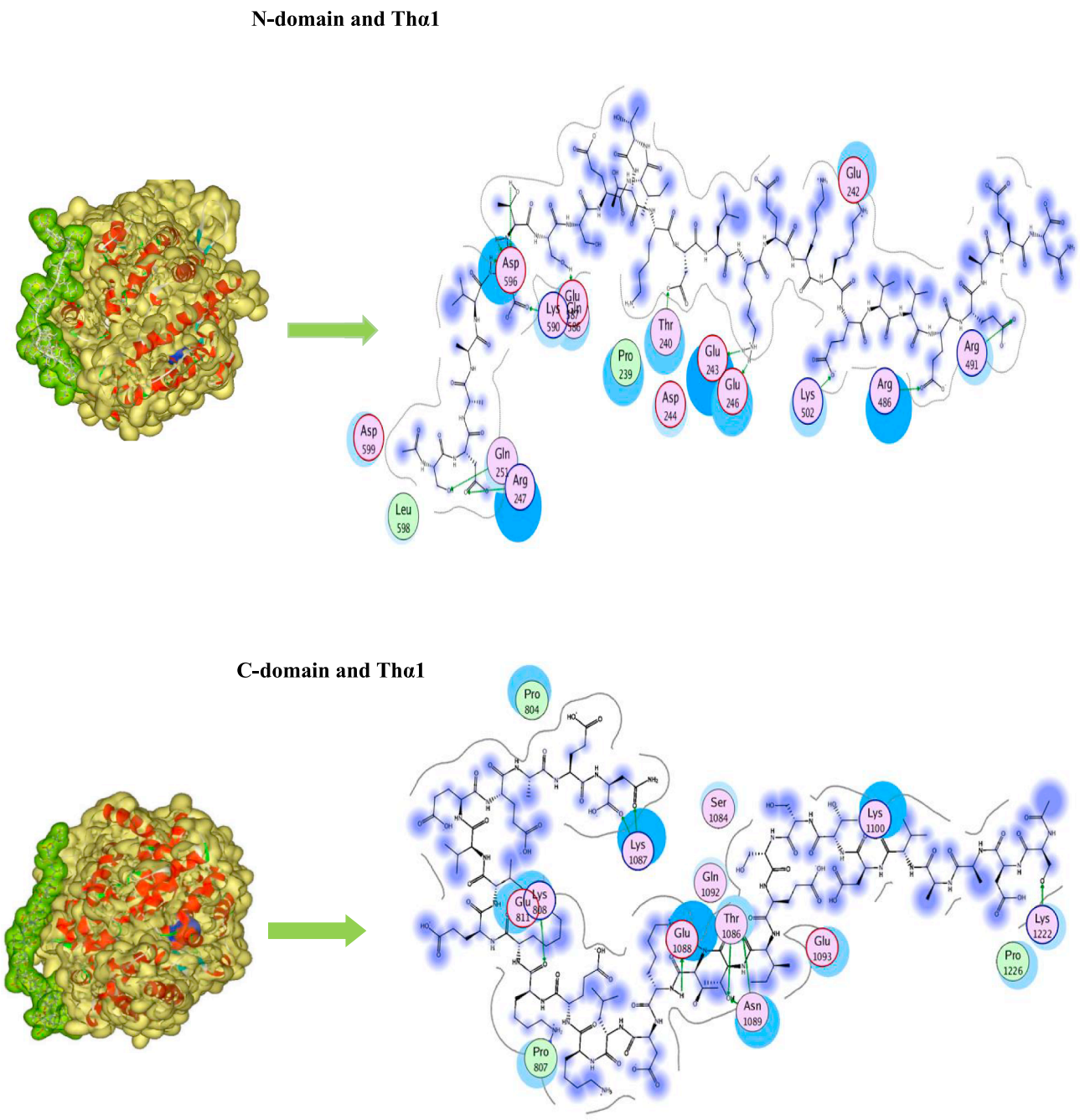


Fig. 4. Molecular modeling of the interaction between Thα1 peptide and ACE. The Thα1 peptide and ACE are shown as CPK (green) and surface mode, respectively. Hydrophobic, polar and acidic residues of ACE enzyme are presented by green, violet and red rings, respectively.

docking between Thα1 peptide and human ACE (PDB ID: 2C6N for *N*-domain and PDB ID: 2OC2 for C-domain) was performed (supplementary data of Figure S1, Table S1 and Table S2). Results showed that the binding energy of human ACE-Thα1 complex for N- and C-domains were −19.87 kcal/mol and 10.63 kcal/mol, respectively. On the base of the results, Thα1 has more affinity for rabbit ACE in comparison with human ACE-domains. The results of molecular mechanism indicated that Thα1 is located in the *N*-domain of ACE by some charged residues (i.e., Glu-242, Glu-243, ASP-244, Glu-246, Arg-247, Arg-486, Arg-491, Lsy-502, Glu-587, Lys-590, Asp-596, and ASP-599), polar residues (i.e., Thr-240, Gln-251, and Gln-586), and hydrophobic amino acids (Pro-239 and Leu-598). In this research, the models showed that Thα1 is surrounded in the C-domain of ACE by residues containing Pro-804, Pro-807, Lys-808, Glu-811, Ser-1084, Thr-1086, Lys-1087, Glu-1088, Asn-1089, Gln-1092, Glu-1093, Lys-1100, Lys-1222, and Pro-1226. The types of interaction between the amino acid residues of Thα1 with ACE residues (N- and C-domain) are shown in Table 2. Our findings demonstrated that in *N*-domain of ACE, Pro-239, and Leu-598 contributes to the interaction through forming hydrophobic interaction. Therefore, hydrophobic forces have a weak role in the forming of the ACE- Thα1 complex. Thr-240, Glu-242, Glu-243, Glu-246 Lys-502, Glu-587, and Asp-596 induce the interaction by constructing a stable hydrogen bond. In addition, electrostatic forces were involved in the interaction of ACE-Thα1 complex by some charged amino acids including Glu-242, Glu-243, Asp-244, Glu-246, Arg-247, Arg-486, Arg-491, Lys-502, Glu-587, Lys-590, and Asp-599. Hence, the electrostatic forces have the most

Table 2
Docking results according to binding energy, hydrophobic interaction, electrostatic interaction and hydrogen bond for ACE- Thα1 complex.

System	Binding energy (kcal/mol)	Hydrophobic interaction	Electrostatic interaction	Hydrogen-bond
ACE- Thα1 (*N*-domain)	− 22.87	Pro-239, Leu-598	Glu-242, Glu-243, Asp-244, Glu-246, Arg-247, Arg-486, Arg-491, Lys-502, Lys-590, Glu-587, Asp-599	Thr-240, Glu-242, Glu-243, Glu-246, Lys-502, Glu-587, Asp-596,
ACE-Thα 1(C-domain)	− 17.74	Pro-804, Pro-807, Pro-1226	Lys-808, Glu-811, Glu-1088, Glu-1093, Lys-1100, Lys-1222	Lys-808, Thr-1068, Lys-1087,Glu-1088, Lys-1222

important role and contribute to the interaction between Thα1 and *N*-domain of the ACE enzyme. The main acting forces between Thα1 and the C-domain of ACE are hydrophobic (i.e., Pro-804, Pro-807, and Pro-1226), electrostatic interactions (i.e., Lys-808, Glu-811, Glu-1088, Glu-1093, Lys-1100, and Lys-1222), hydrogen bond (Lys-808, Thr-1068, Glu-1088, Lsy-1087, and Lys-1222), and Van Der Waals interaction (Ser-1084, Thr-1086, Asn-1089, and Gln-1092) (Table 2). The *N*-domain and C-domain of ACE include Glu amino acid residue at the position of 396 and 993 of the active site, respectively. The distances between Thα1 amino acids residues and Glu-396 are presented in Fig. 5a. In addition, the distance of Ser-2, Val-6, Ser-10, Thr-13, Asp-16, Glu-19, Glu-22, Glu-25, Ala-27, and Asn-29 residues of Thα1 with Glu-993 (posited at the active site of C-domain) are shown in Fig. 5b. The distance of Thα1 residues and the active site of N- and C-domains was more than 20 Å, which refers to not competitive interaction. The results showed that the interaction between Thα1 and ACE was not dominated by S1, S2, and S1′ active site pockets. The interactions of amino acid residues of Thα1 with the ACE-domains are presented in Tables 3 and 4. As shown in Table 3, for *N*-domain, among the 28 amino acids of Thα1 the highest contributions are for Ser-2, Asp-7, Thr-8, Asp-16, Lys-18, Glu-22, and Glu-26. For C-domain, Ser-10, Glu-11, Thr-13, and Lys-20 of the peptide play a more important role in the interaction with ACE enzyme (Table 4). On contrary to our study, Wu et al. identified an ACE inhibitory peptide from sweet sorghum grain hydrolysates with the sequence of TLS. Molecular mechanism of this peptide showed that the ACE inhibitory effect of this peptide is contributed to its C-terminal Ser that can significantly interact with the S1 and S2 active site pockets of the ACE enzyme [68]. Asoodeh et al. demonstrated a novel peptide (i.e., WG-9) from white protein hydrolysate of an ostrich egg. Molecular docking of this peptide indicated that Arg, Glu, Leu, Gly, and Trp residues interact with the active site of the ACE enzyme [69]. Li et al. purified a novel peptide from pistachio hydrolysates (ACKEP). Docking mechanism displayed that ACKEP peptide is surrounded by residues including His-387, Glu-384, Arg-522, Asp-358, Ala-356, and Asn-70. They suggested that the presence of Pro at the C-terminal of ACKEP was similar to the structure of two ACE inhibitory drugs of Enalapril and Lisinopril, which played the main role in the interaction with ACE [70]. Jia et al. showed that KHV tripeptide isolated from *Silkworm pupa* protein effectively interacts with the active site of the ACE with the binding energy of − 52.415 kJ/mol. They demonstrated that this peptide formed a stable hydrogen bond with ACE by Asn-277, Gln-281, Thr-282, His-383, Asp-415, Lys-454, Ser-526, Phe-527, and Gln-530 residues [71]. In a similar study, Tanzadehpanah et al. investigated ACE in the complex with KDEDTEEVP peptide. They suggested that hydrophobic (i.e., Leu-35, Leu-40, Leu-93, Leu-94, Ala-101, and Leu-139) and electrostatic (i.e., Asp-36, Arg-68, Glu-90, Glu-91, Glu-97, Glu-100, and Arg-142) forces have the main role in the interaction between ACE and KDEDTEEVP peptide [72]. Also, this study indicated that the distance of Glu-396 (posited in active site) to the novel peptide was 24.59 Å, which refers to a noncompetitive inhibition [72].

4. Conclusion

Several studies have examined the potential inhibitory effect of natural peptides on ACE activity. In the current study, we investigated the inhibitory potential of Thα1 with the sequence of SDAAVDTSSEITTKDLKEKKEVVEEAEN on ACE activity. In addition, kinetic parameters and the inhibition mechanism (mixed inhibition) of Thα1 were evaluated using Lineweaver-Burk plots. The affinity of Thα1 to N- and C-domains of the ACE enzyme was determined by molecular docking. According to our results, Thα1 demonstrated a potent ACE inhibitory impact with a higher binding affinity to *N*-domain of ACE. Furthermore, the antioxidant activity was determined through different antioxidant assays. Results showed that Thα1 possessed antioxidant activity by ABTS, DPPH, superoxide, and hydroxyl radical scavenging assays as well as reducing cellular ROS levels in 1321N1 human neural

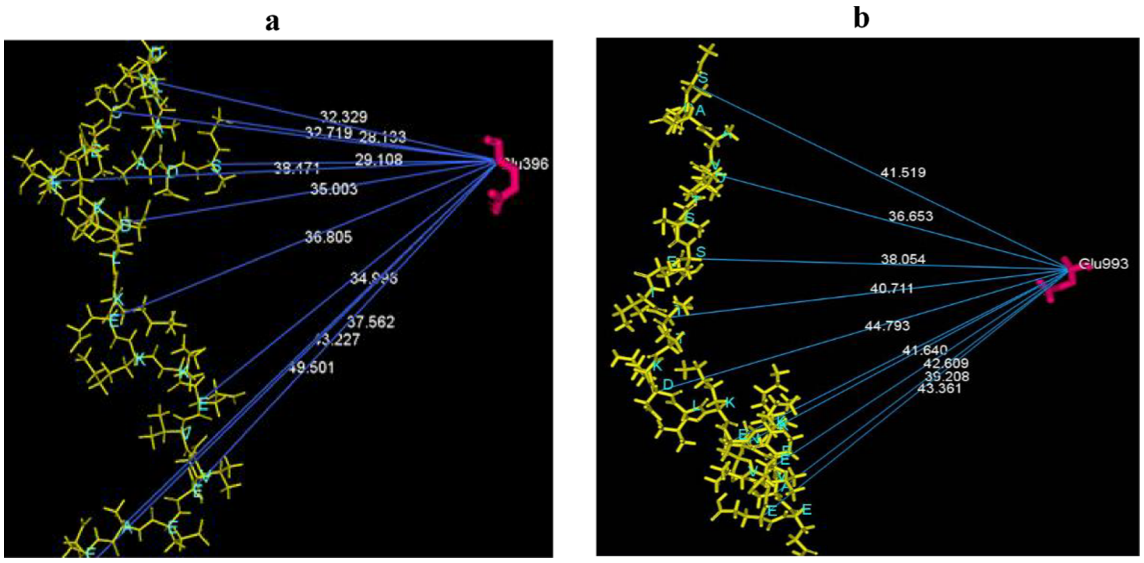


Fig. 5. The distance of several amino acid residues of Thα1 to **(a)** Glu-396 and **(b)** Glu-993 posited at the active site of N- and C-domains of ACE enzyme.

Table 3
The interaction of each residue of peptide (**bold written**) with the *N*-domain of ACE.

ACE-1	Ser-2	Asp-3	Ala-4	Ala-5
Asp-599	Gln-251, Leu-598	Arg-247	not	Asp-596
Asp-7	**Thr-8**	**Ser-9**	**Ser-10**	**Glu-11**
Lys-590, Asp-596	Gln-586, Asp-596	Glu-586	not	not
Thr-13	**Thr-14**	**Lys-15**	**Asp-16**	**Leu-17**
not	not	Pro-239	Thr-240, Asp-244	Arg-247
Glu-19	**Lys-20**	**Lys-21**	**Glu-22**	**Val-23**
Glu-243	Glu-243	Glu-242	Glu-246, Lys-522	not
Glu-25	**Glu-26**	**Ala-27**	**Glu-28**	**Asn-29**
Arg-486	Arg-486, Arg-491	not	not	not
Val-6	**Ilu-12**	**Lys-18**	**Val-24**	
Asp-596	not	Glu-243, Glu-246	not	

Table 4
The interaction of each residue of Thα1 (**bold written**) with the C-domain of ACE.

ACE-1	Ser-2	Asp-3	Ala-4	Ala-5
not	Lsy-1222	Pro-1226	not	not
Asp-7	**Thr-8**	**Ser-9**	**Ser-10**	**Glu-11**
not	Glu-1093	not	Ser-1084,Asn-1089,Glu-1093	Asn-1089,Gln-1092,Glu-1093
Thr-13	**Thr-14**	**Lys-15**	**Asp-16**	**Leu-17**
Thr-1086,Glu-1088Asn-1089	Glu-1088	Glu-1088	not	Lys-1087
Glu-19	**Lys-20**	**Lys-21**	**Glu-22**	**Val-23**
Glu-243	Pro-807,Lys-808, Glu-811	Lys-808, Glu-811	not	not
Glu-25	**Glu-26**	**Ala-27**	**Glu-28**	**Asn-29**
not	not	not	Pro-804, Pro-807	Lys-1087
Val-6	**Ilu-12** Asn-	**Lys-18**	**Val-24**	
Lys-1100	1089	not	not	

astrocytoma cells. In conclusion, based on our findings Thα1 exhibited ACE inhibitory and antioxidant properties as a source of natural peptides. However, further studies are needed to evaluate *in vivo* antioxidant or antihypertensive ability of Thα1.

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Conflict of Interest and Ethical Standards

Authors declare that they have no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.04.003.

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