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Inhibition effect of Gly-Arg-Gly-Asp-Ser (GRGDS) and Arg-Gly-Asp (RGD) Bioactive Peptides on Angiotensin-Converting Enzyme Activity Purified from Human Serum

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Highlights:

- Angiotensin converting enzyme
- Purification Inhibition

Keywords:

- Angiotensin-converting enzyme (ACE)
- bioactive peptides
- Gly-Arg-Gly-Asp-Ser
- Arg-Gly-Asp
- Inhibition Purification

ABSTRACT:

Angiotensin-converting enzyme (ACE, EC 3.4.15.1) is a physiological target for researching new antihypertensive drugs, as it is a substantial enzyme in the regulation of blood pressure. Herein, ACE was purified from human serum with affinity chromatography. V_{max} and K_M values were found as 60.98 (µmol/min)/mL and 0.34 mM, respectively. The effects of Gly-Arg-Gly-Asp-Ser (GRGDS) and Arg-Gly-Asp (RGD) bioactive peptides on purified ACE were researched. Also, captopril, a specific ACE inhibitory, was used as a reference inhibitor. Bioactive peptides, GRGDS and RGD, demonstrated the inhibitory effect on purified ACE with IC_{50} values of 46.39 µM and 456.46 µM, respectively. K_i values and kind of inhibition for GRGDS and RGD by the Lineweaver-Burk chart were found. The kind of inhibitory for these bioactive peptides was calculated as reversible-competitive inhibitory. K_i values for GRGDS and RGD were obtained as 93.28 µM and 435.67 µM, respectively. The IC_{50} value of captopril was designated as 1.57 nM. The inhibitory kind of captopril was designated as reversible non-competitive inhibitory and the K_i value was 0.99 nM. In this study, it was concluded that RGD and GRGDS bioactive peptides have the potential to be utilized as ACE inhibitors.

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INTRODUCTION

Hypertension, which influences millions of people around the world, is an important risk reason for many illnesses like chronic kidney diseases, stroke, and heart failure (Bondre et al., 2020). The angiotensin-converting enzyme is a metal carboxypeptidase that regulates blood pressure by hydrolyzing decapeptide angiotensin I (Ang I) to octapeptide angiotensin II (Ang II). Also, in the kallikrein kinin system (KKS), ACE ineffectives bradykinin, a potent vasodilator. High ACE activity causes overproduction of the vasoconstrictor Ang II, which leads to hypertension (Lavoie and Sigmund, 2003). Therefore, inhibition of ACE is one of the most significant goals of treating hypertension. ACE inhibitors are extensively utilized to check hypertension. However, long-time utilization of these inhibitors can cause very adverse effects like cough, headache, hyperkalemia, and edema (Hermida et al., 2011).

Bioactive peptides are specific protein components that can affect human health and have an affirmative effect on body functions (Kitts and Weiler, 2003). Bioactive peptides play a significant role in human health as they take part in the metabolic functions of living organisms (Sánchez and Vázquez, 2017). It has been determined that these compounds have antioxidant, antimicrobial, antithrombotic, antihypertensive, opioid, and immunomodulatory properties (Rezzani et al., 2010; Lin et al., 2017). Recently, as a result of research on bioactive peptides, it has been observed that these compounds are efficient in the intercepting and treatment of many illnesses. Therefore, attention to the production and properties of bioactive peptides has increased remarkably in the last few years (Sánchez and Vázquez, 2017). The inhibition effect of many bioactive peptides obtained from natural sources on ACE has been investigated. In a study by Zhang et al., two new ACE inhibitory peptides Leu–Gly–Val–Pro (LGVP) and Val–Thr–Tyr (VTY) were identified from *Spirulina platensis* protein hydrolysates, and their IC_{50} values were obtained to be 45.76 μM and 23.39 μM, respectively (Zhang et al., 2022). Seven new ACE inhibitors have been identified from the *Channa striatus*. Among these peptides, LPGPGP and EYFR peptides demonstrated the strongest ACE inhibitory impact by IC_{50} values of 186.3 μM and 179.2, respectively ($P>0.05$) (Ma et al., 2021). Bioactive peptides are a more effective alternative to antihypertensive synthetic drugs because they are natural.

Bioactive peptides are compounds with great potential for the intercept and cure of hypertension. In many clinical and experimental studies, it has been designated that bioactive peptides show strongly in vivo antihypertensive effects in both human and animal models (Majumder and Wu, 2014). For example, in a clinical study, little dosages (2.0–10.2 mg/day) of the tripeptides Valine-Proline-Proline (VPP) and Isoleucine-Proline-Proline (IPP) derived from milk casein were observed to reduce diastolic blood pressure (DBP) by -1.9 mmHg (95% CI -3.1 to -0.8 mmHg, $P < 0.001$) and systolic blood pressure (SBP) by -4.0 mmHg (95% CI -5.9 to -2.1 mmHg, $P<0.001$) in mildly hypertensive human subjects (Turpeinen et al., 2013). In another study, the oral antihypertensive effect of IAK, YAKPVA, LVYPFTGPIPN, HLPLP, and WQVLPNAVPAK peptides isolated from casein fractions was determined in spontaneously hypertensive rats (SHR). DBP and SBP of the rats were evaluated using the tail-cuff process before the implementation and also at 2, 4, 6, 8, and 24 hours after the administration. These peptides significantly lowered DBP and SBP in SHR (Miguel et al., 2010).

The RGD bioactive tripeptide is the cell binding site of numerous blood, adhesive extracellular matrix (ECM), and cell surface proteins. This bioactive peptide is the major integrin-binding domain found in ECM proteins like vitronectin, fibrinogen, fibronectin, bone sialoprotein, and osteopontin (Arnaout et al., 2005). RGD peptide, which plays a significant role in cell recognition and cell adhesion, has been utilized in tissue engineering and tumor treatment with some chemical and

processes recombinant means (Wang et al., 2013). At the same time, RGD peptide has been observed to have a therapeutic effect on pulmonary hypertension and obstructive pulmonary disease (Welschoff et al., 2014).

The GRGDS bioactive pentapeptide was identified as a cell-binding protein domain originating from the cell-connected domain of fibronectin. Osteopontin uses this peptide motif for cell adhesion. Also, the GRGDS bioactive peptide contains the RGD bioactive peptide sequence found in many ECM adhesive proteins (Miyamoto et al., 1995). In a study, the combination of GRGDS+GPRP peptides synergistically inhibited platelet aggregation in plasma. Therefore, it has been observed that the combination of these peptides can be used in antithrombotic therapy (Adelman et al., 1990).

In this work, the inhibitory impact of Gly-Arg-Gly-Asp-Ser and Arg-Gly-Asp bioactive peptides on ACE purified from human serum was explored. These bioactive peptides showed a substantial in vitro inhibitor impact on purified ACE. Also, the IC_{50} value, the K_i value, and the inhibition kind of captopril, specifically an inhibitory, on purified ACE were found. The impact of GRGDS and RGD bioactive peptides on ACE from human serum has not been researched so far.

MATERIALS AND METHODS

Materials

RGD peptide, GRGDS peptide, lisinopril, captopril, N-[3-(2-Furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG), sodium tetraborate ($Na_2B_4O_7.10H_2O$), and HepesNa were bought from Sigma-Aldrich.

Obtain the human serum

To achieve human serum, blood was obtained from the Van Red Crescent Blood Center in Turkey. The collected blood was left for half an hour to coagulate and then poured into centrifuge tubes. Human blood was centrifuged at 2500 x *g* for 5 min at chamber temperature. After centrifugation, two phases were formed, the upper phase being yellow-colored serum and the lower part being dark-colored erythrocytes. The serum was attentively divided from the blood and kept in the freezer for utilization in subsequent kinetic studies (Thavasu et al., 1992).

Purification process by the affinity chromatography

NHS (N-hydroxysuccinimide)-activated Sepharose 4 Fast Flow was taken and acquired utilizing the manufacturer's specified procedure. First, the affinity gel was cleansed with cold 1 mM HCl. Subsequently, a coupling buffer (5 mM Lisinopril, 0.2 M $NaHCO_3$, and 0.5 M NaCl) was added to the affinity gel. This mixture was incubated overnight at 4 °C to bind lisinopril to the affinity gel. The affinity gel was appended to a beaker including 0.1 M Tris-HCl (pH 8.5) buffer and the admixture was carefully stirred for several clocks to avoid unreacted groups on the affinity gel. After this treatment, the affinity gel was cleansed 3 times with a Tris-HCl buffer (pH 8.5, 0.1 M). Next, the affinity gel was cleansed 3 times with an acetate buffer (pH 4.5, 0.1 M).

The affinity gel was appended onto the column (1 cm x 10 cm) with equilibration and cleaning buffer (pH 8.0, 0.3 M NaCl, and 20 mM Tris). The flow rate was adjusted to 40 mL/h utilizing a peristaltic pump to cleanse and equilibrate the affinity column. Human serum was loaded onto the affinity column and then again the column was cleansed by an equilibration buffer. Cleaning of the gel was followed by absorbance at 280 nm and washing continued until the final absorbance was 0.1. Then, elution was taken by passing $Na_2B_4O_7.10H_2O$ (pH 9.0, 50 mM) elution buffer with a peristaltic pump. Elutions were taken in 1.5 mL portions. Pure human serum ACE activity in the portions was determined at 345 nm. The purified ACE was aliquoted and stored in the freezer (Sabeur et al., 2001).

ACE Activity designation

The ACE activity was determined as the decrease in absorbance at 345 nm and 35 ºC. The assay cuvette comprised 50 mM HepesNa buffer (pH 7.5, 10 μM $ZnCl_2$, and 0.3 M NaCl), the ACE, and 1 mM FAPGG (Holmquist et al., 1979).

Protein designation

The protein concentrations of the pure portions and the human serum were determined with Coomassie Brillant Blue G-250 at 595 nm (Bradford, 1976).

Molecule weight designation by SDS-PAGE

The purity of ACE was identified with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). At the same time, the purity and molecule weight of ACE purified from human serum were characterized by utilizing the SDS-PAGE process. Here, stacking (4% acrylamide) gel and running (10% acrylamide) gel inclosing 1% SDS were made according to the Laemmli process (Laemmli, 1970).

Preparation of RGD, GRGDS bioactive peptides, and captopril solution

2 mg of Arg-Gly-Asp peptide was disentangled in little pure water and it was fulfilled to 1 mL utilizing pure water.

1 mg of Gly-Arg-Gly-Asp-Ser peptide was disentangled in little pure water and it was fulfilled to 1 mL utilizing pure water.

0.001 mg of captopril was disentangled in little pure water and it was fulfilled to 50 mL utilizing pure water.

In vitro inhibitory impacts of GRGDS and RGD bioactive peptides on ACE

The effects of RGD and GRGDS bioactive peptides on ACE from human serum were researched. Bioactive peptides at various concentrations were attached to the appraisal tube (50 mM HepesNa buffer,1 mM FAPGG, 100 μL purified ACE) to define the concentration range and ACE activities. Using these concentrations were drawn % activity against inhibitory concentration graphs. Lineweaver-Burk plots were plotted with five different concentrations of FAPGG and three different concentrations of bioactive peptides. Inhibitory kind and K_i constants of RGD and GRGDS bioactive peptides were determined from these charts. At the same time, IC_{50}, K_i values, and inhibition kind of captopril, a specific ACE inhibitor, were found (Lineweaver and Burk, 1934).

RESULTS AND DISCUSSION

ACE is an important enzyme that plays a role in the control of blood pressure by transforming Ang I to Ang II, a vasoconstrictor, due to its participation in the renin-angiotensin system (Perazella et al., 2003). ACE was purified from human serum by affinity chromatography process in one step utilizing NHS (N-hydroxysuccinimide)-activated Sepharose 4 Fast Flow column filler. A specific ACE inhibitory, lisinopril, was utilized as a ligand. The inhibitory impact of some bioactive peptides on the pure ACE was examined.

There are two kinds of ACE: the high molecule weight somatic kind (130-180 kDa) and the smaller molecule weight germinal kind (90-100 kDa) found only in the testis (Bernstein et al., 2018). In a study, the ACE was obtained from rabbit testicles and the molecule weight of this enzyme was detected as 100 kDa (El-Dorry et al., 1982). In another study, only one form (180 kDa) of the ACE was obtained from the pig kidney, and merely two kinds of the ACE (170 kDa and 180 kDa) obtained from the pig striatum were purified by affinity chromatography (Hooper and Turner, 1987). Herein, the

purity and molecule weight of the ACE purified from human serum were described with SDS-PAGE, and two bands, 60 kDa and 70 kDa were seen on the gel (Figure 1). At the same time, in our former works, the molecule weights of the ACE obtained from sheep kidneys, human plasma, and sheep lungs were detected to be 60 and 70 kDa (Bas, 2021; Basi and Turkoglu, 2018; Aydin et al., 2021). Here, the molecule weight revealed by the SDS-PAGE method is the molecule weight of the subunits of the ACE. In our study, it was observed that ACE from human serum is a dimer enzyme consisting of two subunits.

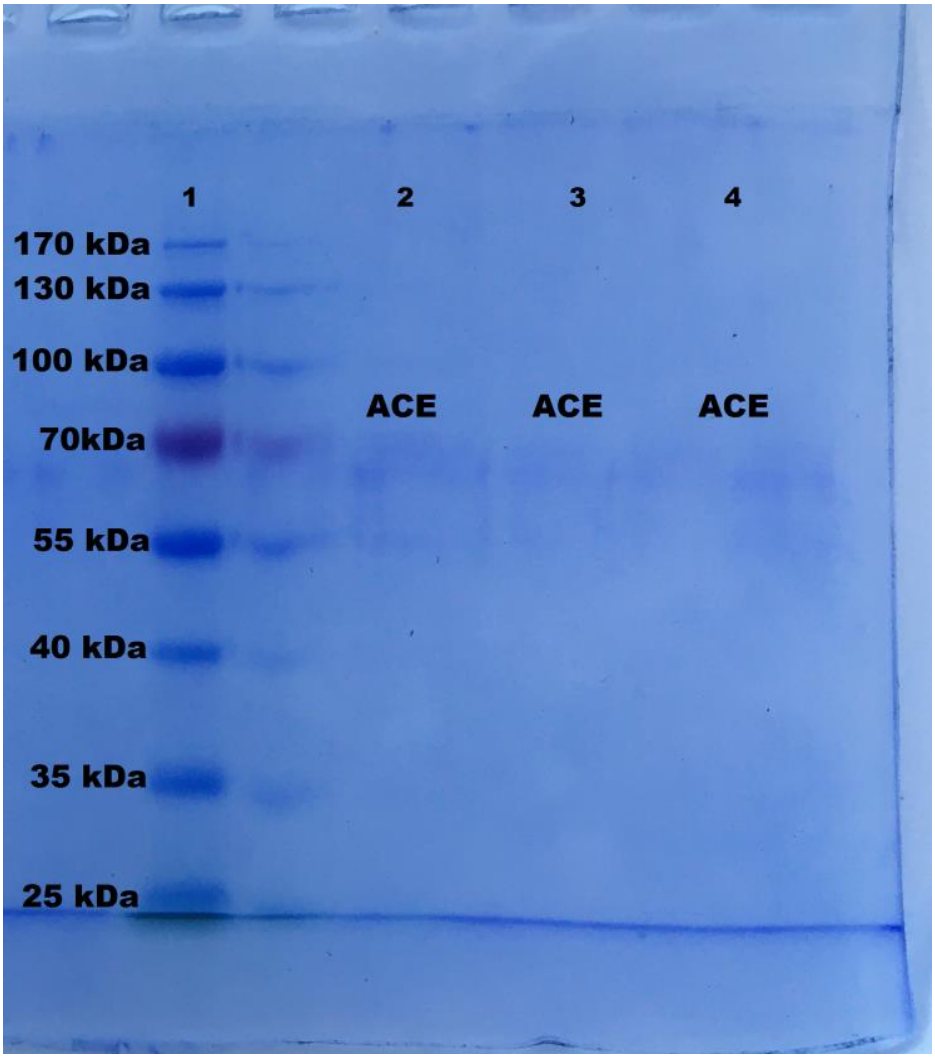

Figure 1. SDS-polyacrylamide gel electrophoresis of ACE purified by affinity chromatography. Lane 1: Standard proteins (Fermentas unstained protein ladder SM0671). Lanes 2, 3, and 4: purified angiotensin-converting enzyme (ACE) from human serum

Biochemical features were designated to characterize the ACE obtained from human serum. Assessments were taken using 5 different concentrations of FAPGG for the pure ACE. Assessments were performed for the ACE at 5 dissimilar FAPGG values. Then, the Lineweaver-Burk graphic with these values was drawn. V_{max} and K_M constants from this graphic were found to be 60.98 (μmol/min)/mL and 0.34 mM, respectively (Figure 2).

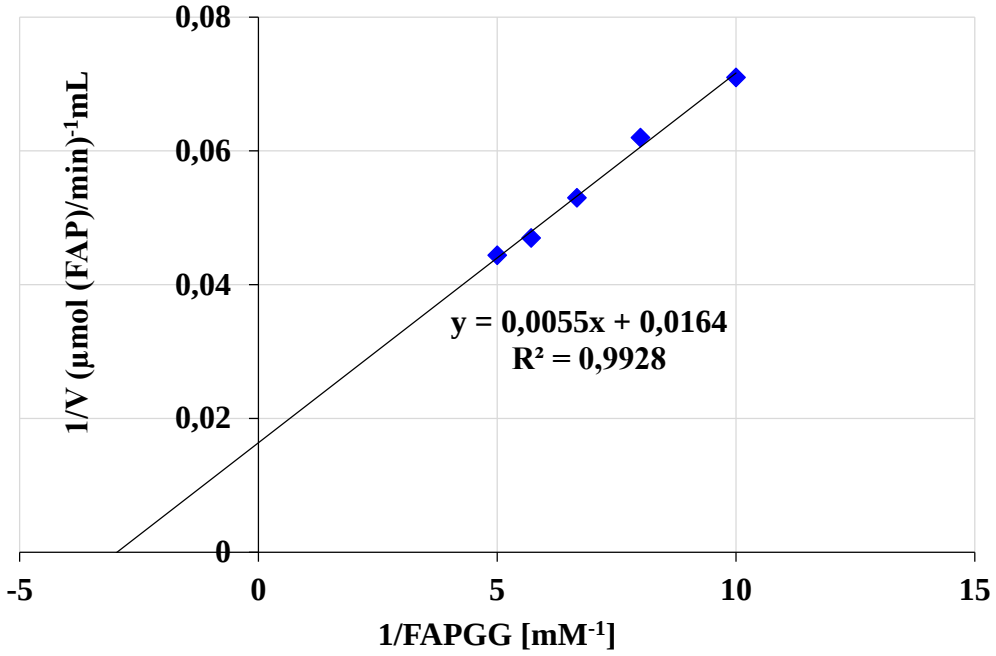

Figure 2. Lineweaver–Burk graph with five dissimilar substrate concentrations (FAPGG) used for the definition of V_{max} and K_M

High ACE activity is one of the causes of hypertension. As a result, blood pressure rises too much. ACE inhibitors like captopril, enalapril, and fosinopril are generally utilized in the cure of hypertension. However, these inhibitors have been determined to have too many adverse impacts. That's why, in recent studies, the inhibitory effect of bioactive peptides, which are known to be more natural and have fewer adverse effects, on ACE has been determined. In a work by Liang et al., a tripeptide IAF with admissible bioavailability features was identified and this peptide demonstrated a substantial inhibitory impact on ACE with an IC_{50} constant of 19.87 ± 0.50 µM (Liang et al., 2021). In our former work, glutathione (GSH) peptide and lisinopril inactivated ACE purified from human plasma. IC_{50} and K_i values for GSH and lisinopril were found as 16.2 µM and 0.781 nM, respectively. K_i values of these compounds were 11.7 µM and 0.662 nM, respectively. The kind of inhibitory of these compounds was identified as reversible non-competitive (Basi and Turkoglu, 2019). In our former work, the IC_{50} values of fosinopril, captopril, and lisinopril, which show an inhibitor impact on ACE purified from bovine lung, were determined as 1.159 µM, 0.835 nM, and 4.085 nM, respectively (Karahan and Turkoglu, 2021). Two novel ACE inhibitor peptides (CDF and EACF) were determined from rabbit meat proteins. The IC_{50} values of CDF and EACF peptides were 192.17 ± 2.46 µM and 41.06 ± 0.82 µM, respectively. The kind of inhibitory of these peptides was defined to be competitive and non-competitive, respectively (Chen et al., 2022). It has been observed that bioactive peptides, which have an inhibitory impact on ACE, are agents that can be used instead of synthetic inhibitors in diseases like hypertension, atherosclerosis, and cardiovascular diseases.

Many studies have researched ACE inhibitors from natural resources such as peptides, antioxidant compounds, and vitamins with fewer side effects. For example, in one work, two ACE inhibitor peptides QLLLQQ and TVGMTAKF were identified in horse gram flour. The IC_{50} values of these peptides were 75.0 µM and 30.3 µM, and K_i values were 0.18 µM and 0.01 µM, respectively. The inhibitory kind of QLLLQQ and TVGMTAKF peptides was designated as reversible competitive from the Lineweaver-Burk chart (Bhaskar et al., 2019). In our previous work, reduced glutathione

peptide, vitamin B_3 (nicotinamide), and reduced nicotinamide adenine dinucleotide (NADH) compounds showed inhibitory effects on ACE purified from sheep kidneys. IC_{50} constants for these compounds were designated as 14.3 µM, 7.3 µM, and 244.33 µM, respectively and K_i values were 15.4 µM, 6.7 µM, and 175.08 µM, respectively. The inhibition kind of these compounds was designated as reversible non-competitive (Bas, 2021; Kiylik et al., 2022). The butanol and water extracts of the *Matricaria chamomilla* L. indicated an inhibitory impact on ACE purified from human plasma (Bas et al., 2022).

Herein, the inhibitory impact of GRGDS and RGD bioactive peptides on pure ACE was researched. GRGDS and RGD peptides demonstrated a significant inhibitory impact with IC_{50} values of 46.39 µM and 456.46 µM, respectively (Figures 3, 4). The IC_{50}, inhibition kind, and K_i values of captopril on ACE from these charts were defined. The IC_{50} value of captopril was calculated to be 1.57 nM (Figure 5). K_i values and inhibitory kinds of these bioactive peptides were identified from Lineweaver-Burk charts. The kind of inhibitory for RGD and GRGDS bioactive peptides was identified as reversible competitive inhibitory. K_i values for GRGDS and RGD bioactive peptides were calculated as 93.28 µM and 435.67 µM, respectively (Figures 6, 7). The natural substrate of the ACE enzyme, Ang I, is a decapeptide. Since the compounds we used in this study are peptides, they showed a reversible competitive inhibition effect as expected. The inhibitory kind and K_i value of captopril were calculated to be non-competitive and 0.99 nM from the Lineweaver-Burk chart (Figure 8) (Table 1). In many works, it has been determined that the thiol-including captopril inhibitor has a non-competitive inhibition effect by forming powerful bonds with zinc, which is near the active site of the ACE. Also, although inhibitors like enalaprilat, ramiprilat, and captopril are described to be competitive inhibitors, some works have indicated non-competitive and mixed inhibitory impacts (Baudin and Bénéteau-Burnat, 1999). Here, it was detected that GRGDS and RGD bioactive peptides, which indicate an inhibitory impact on ACE, may have both therapeutic effects and protection against hypertension.

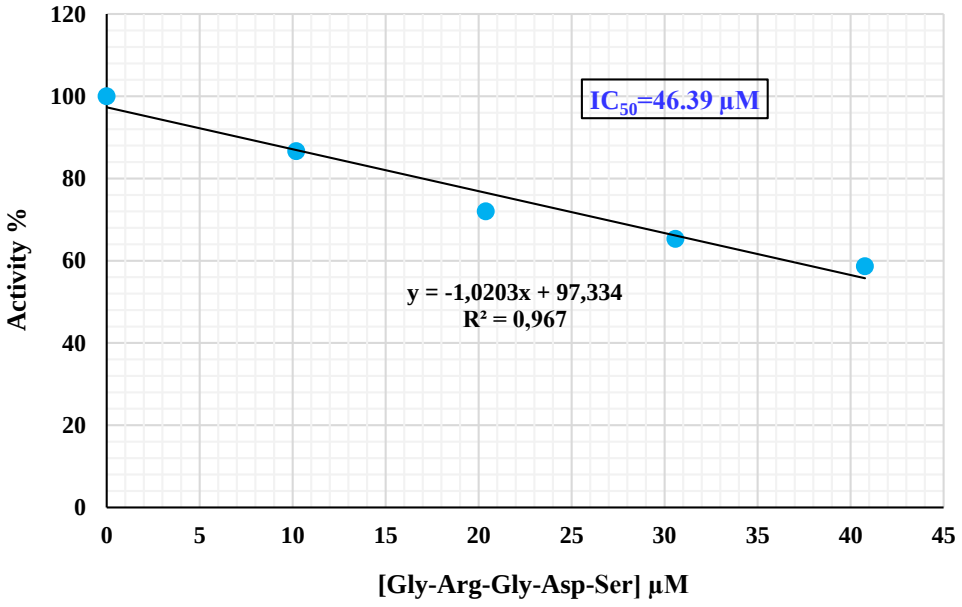

Figure 3. The inhibitory impact of Gly-Arg-Gly-Asp-Ser (GRGDS) bioactive peptide on ACE from human serum. Four dissimilar GRGDS peptide (from 10.20 to 40.78 µM) concentrations on ACE activity were explored

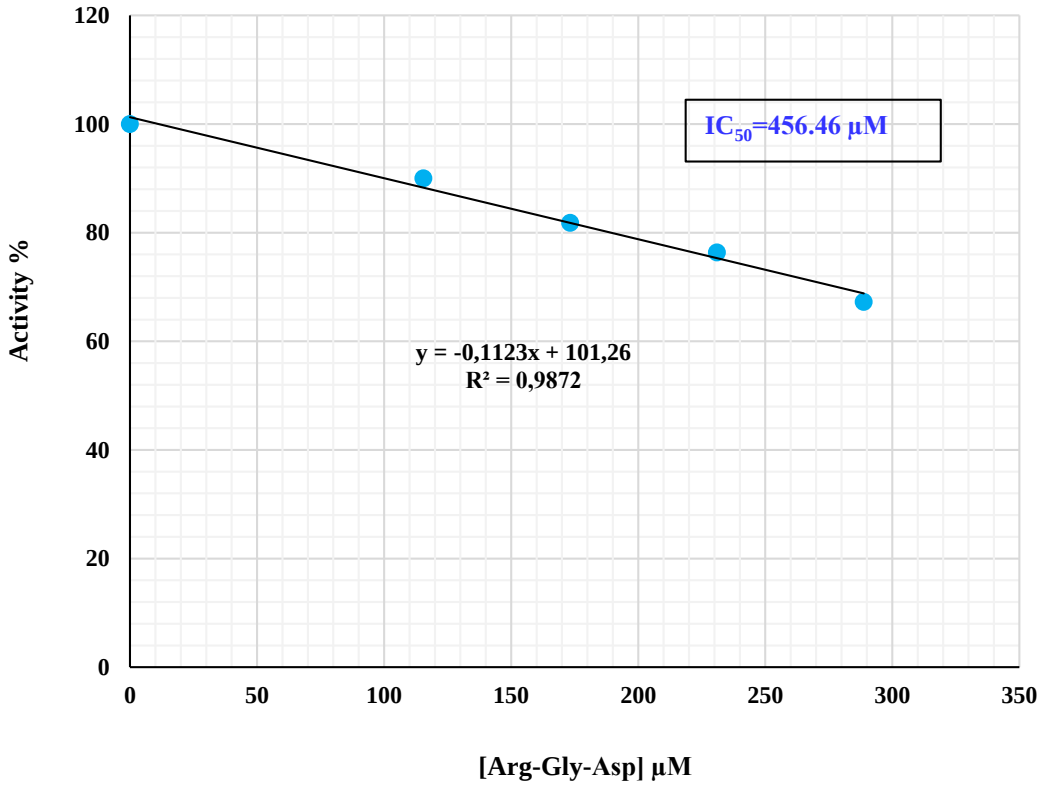

Figure 4. The inhibitory impact of Arg-Gly-Asp (RGD) bioactive peptide on ACE from human serum. Four dissimilar RGD peptide (from 115.5 to 288.75 µM) concentrations on ACE activity were explored

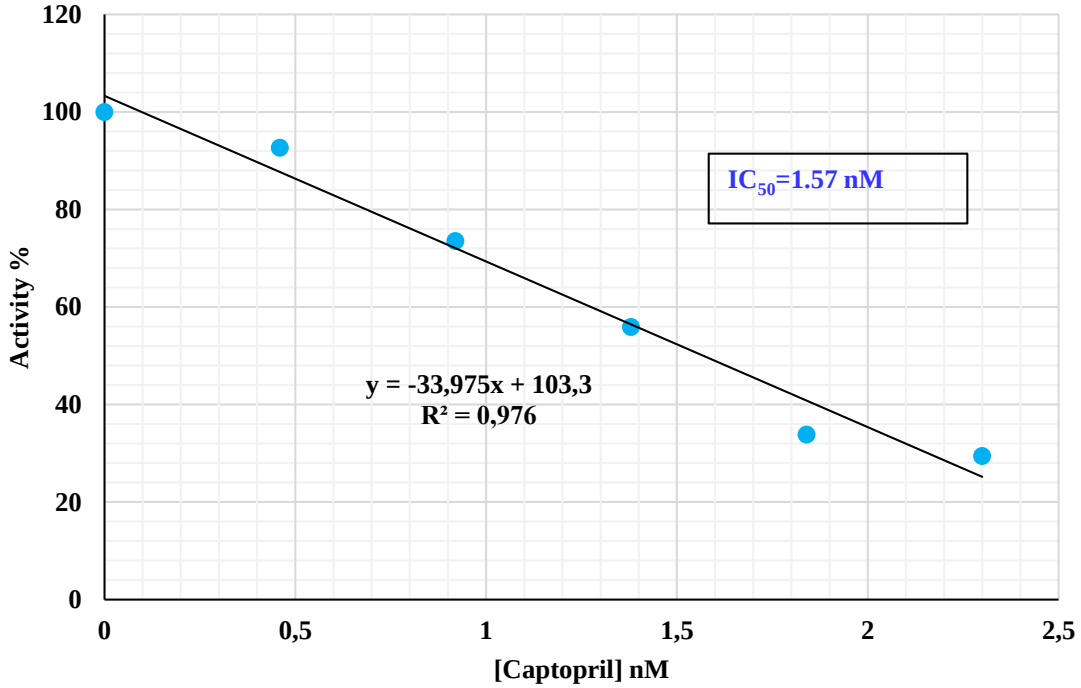

Figure 5. The inhibitory impact of captopril on ACE from human serum. Five dissimilar captopril (from 0.46 to 2.30 nM) concentrations on ACE activity were explored

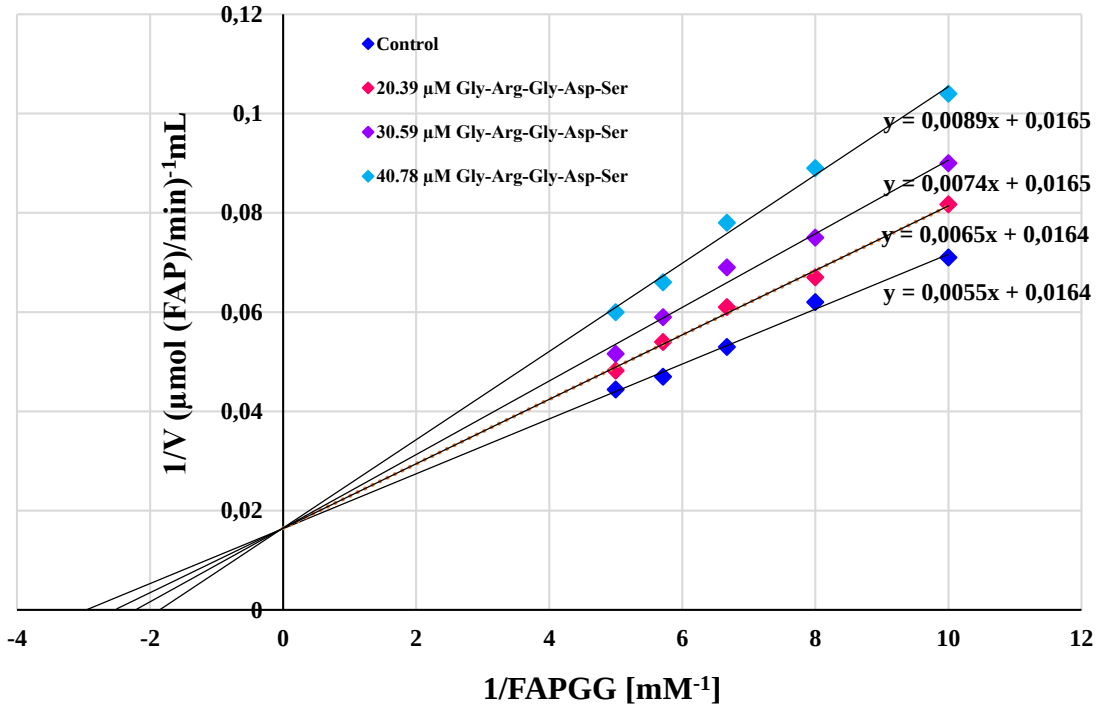

Figure 6. Lineweaver–Burk graph with five dissimilar substrate concentrations (FAPGG) and three dissimilar Gly-Arg-Gly-Asp-Ser (GRGDS) bioactive peptide concentrations used for the definition of inhibitory kind and K_i

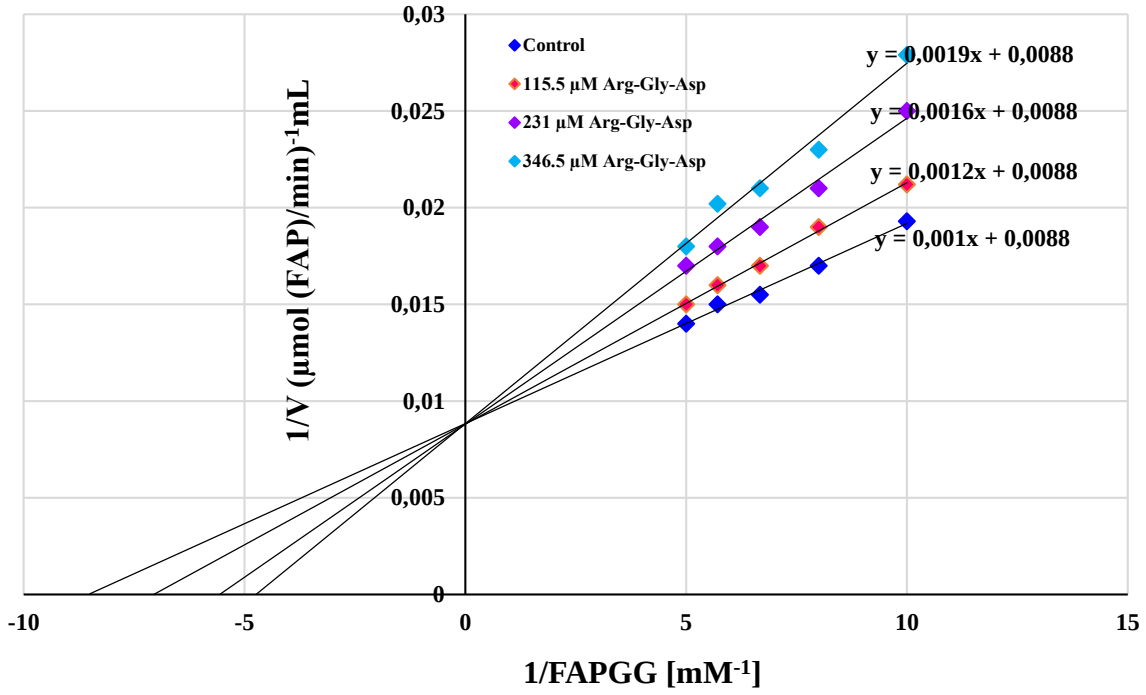

Figure 7. Lineweaver–Burk graph with five dissimilar substrate concentrations (FAPGG) and three dissimilar Arg-Gly-Asp (RGD) bioactive peptide concentrations used for the determination of inhibitory kind and K_i

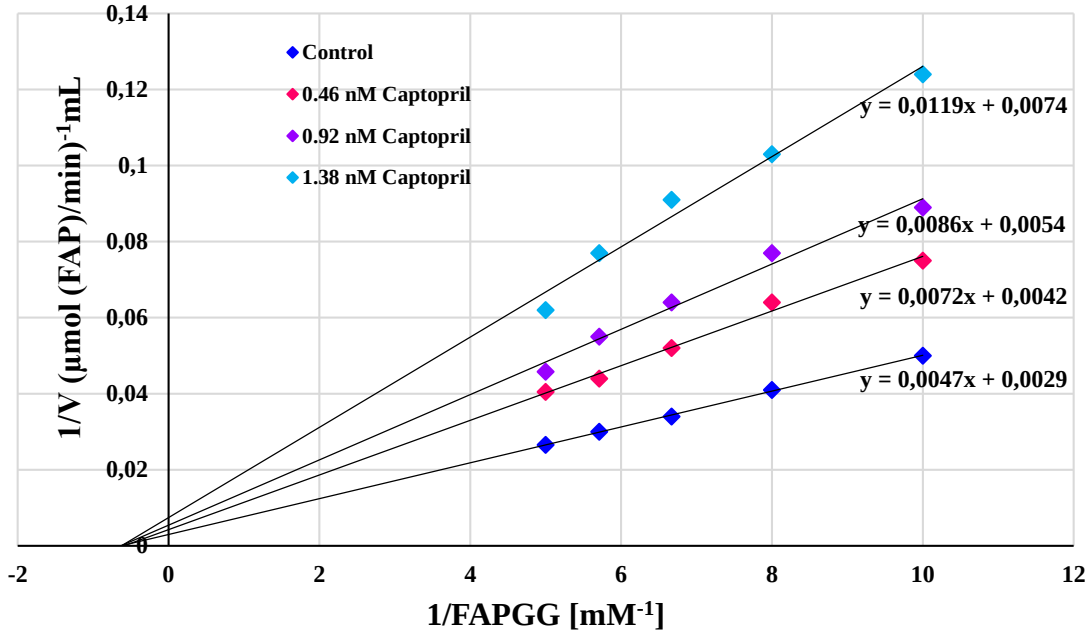

Figure 8. Lineweaver–Burk graph with five dissimilar substrate concentrations (FAPGG) and three dissimilar captopril concentrations used for the determination of inhibitory kind and K

Table 1. IC_{50}, K_i values, and inhibition types were obtained from regression analysis graphs for the ACE in the presence of different Arg-Gly-Asp (RGD), Gly-Arg-Gly-Asp-Ser (GRGDS) bioactive peptides, and captopril concentrations

ACE Inhibitory	IC_{50}	K_i	Inhibitory kind
GRGDS peptide	46.39 µM	93.28 µM	Reversible Competitive
RGD peptide	456.46 µM	435.67 µM	Reversible Competitive
Captopril	1.57 nM	0.99 nM	Reversible Non-competitive

In many in vivo works, it has been detected that various bioactive peptides can significantly decrease blood pressure with ACE inhibition upon oral or intravenous implementation (Majumder and Wu, 2014; Balti et al., 2012). In one study, a tri-peptide IRW was identified from the thermolysin-pepsin hydrolyzate of the egg white protein ovotransferrin, which showed a potent inhibitory effect on the ACE. This peptide significantly decreased SBP by 40 mmHg in SHR after 18 days of therapy at a dose of 15 mg/kg body weight. It has also been observed to decrease plasma Ang II levels simultaneously, possibly by ACE inhibition (Majumder et al., 2013). In another study, the milk-derived peptides AYFYPEL and RYLGY from bovine casein hydrolyzate showed ACE inhibitory and antioxidant effects in vivo. Oral implementation of these peptides to SHR at a dosage of 5 mg/kg BW has been found to significantly lower blood pressure (Contreras et al., 2009). A new peptide (GHS or Gly-His-Ser) isolated from the 3 kDa membrane ultrafiltration permeate of a pancreatin+pepsin rapeseed protein showed an inhibition effect on ACE, with an IC_{50} value of 0.52 ± 0.01 mg/mL. Oral implementation of this peptide to SHR (30 mg/kg body weight) resulted in a maximal blood pressure decrease of -17.29 ± 2.47 mmHg after 6 hours (He et al., 2013).

The RGD bioactive peptide is a cell adherence motif displayed in plasma proteins and many ECM. The RGD is the most studied adhesive peptide in the field of biomaterials. RGD peptide is widely utilized in many different pathological and physiological procedures in the therapy of tumors, the improvement of antithrombotic medicaments, and tissue engineering (Wang et al., 2013; Colombo

and Bianchi, 2010). The GRGDS bioactive peptide contains the RGD peptide, which is a recognition site in interactions between cell membrane receptors and ECM molecules**.** Recent studies have shown that RGD and GRGDS bioactive peptides inhibit angiogenesis and platelet aggregation (Nicosia and Bonanno, 1991). In a study on mice, it was reported that RGD peptide may be effective in the treatment of hypertension and asthma by showing a relaxing effect on the pulmonary arteries and airways (Welschoff et al., 2014)

CONCLUSION

Hypertension is a chronic health issue that leads to illnesses like atherosclerosis, heart disease, stroke, aneurysm, and kidney failure. ACE inhibitors, which cause a reduction in arterial blood pressure, are usually used in patients with hypertension. However, it has been observed that synthetic drugs like captopril, enalapril, lisinopril, and fosinopril have many adverse effects after long-term use. Since these synthetic drugs are cyclic compounds in their molecular structures, they are difficult to detoxify and excreted in metabolism, and therefore they have too many side effects. It has been observed in recent years that bioactive peptides acquired from natural sources have an inhibitory impact on ACE. Therefore, studies on these peptides have been increasing recently. In many in vivo studies, it has been found that these peptides have antihypertensive effects with oral and intravenous administration of many bioactive peptides. Herein, the ACE was successfully purified from human serum in the only step. The inhibition effect of RGD and GRGDS bioactive peptides on the purified ACE was researched and an important inhibitory effect was found. It has been determined that RGD and GRGDS bioactive peptides, which have specific binding sites between integrins and their ligands, have a strong potential in the treatment of illnesses like cancer, angiogenesis, asthma, cardiovascular disease, and hypertension. In our study, it was concluded that these bioactive peptides have the potential to be utilized to be an ACE inhibitor when compared with a specific ACE inhibitor, captopril.

Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

Vedat Türkoğlu: Supervision, Conceptualization, and Project Administration. Vedat Türkoğlu, Resul Adanaş, and Zehra Baş: Data curation, Writing-original draft preparation. Resul Adanaş and Zehra Baş: Performed the experiments. Vedat Türkoglu and Zehra Baş: Visualization, Investigation, Writing-review & editing.

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