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Molecular design and chemical synthesis of peptide inhibitors of Angiotensin I converting enzyme (ACE) for prevention and therapy of hypertension

B. K. Yakimova*, D. Petkova and I. B. Stoineva

Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad.G. Bonchev str. bl.9, 1113 Sofia, Bulgaria

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Angiotensin I-converting enzyme plays a central role to the regulation of renin-angiotensin system (RAS). The inhibitors of ACE are an attractive target for drug design due to its critical role in hypertension and cardiovascular diseases. The creation of new more effective inhibitors of ACE with less undesirable side effects, requires the search for new molecular and synthetic design of desired compounds. Another focus is development of functional foods as a possible prophylaxis for hypertension.

The targeted short peptides -H-Ile-Ala-Pro-OH, H-Leu-Ala-Pro-OH, H-Val-Ala-Pro-OH and H-Val-Ala-Trp-OH were synthesized by Fmoc–SPPS strategy. The newly synthesized peptides were purified by HPLC and characterized by UPLC and NMR analysis. For determination of ACE inhibitory activity was used modified method of *Jimsheena and Gowda*, based on interaction between ACE and the specific synthetic substrate hippuryl-histidyl-leucine (HHL). Chemically synthesized tripeptides exhibit inhibitory activity comparable to that of a commercially used in medicine lisinopril

Keywords: cardiovascular diseases (CVD), renin-angiotensin system (RAS), angiotensin-I converting enzyme (ACE), inhibitors of ACE

INTRODUCTION

Angiotensin I converting enzyme (ACE) is a zinc and chloride dependent dipeptidyl carboxypeptidase. Its main function is the activation of the renin- angiotensin system (RAS) [1,2] by catalyzing the cleavage of two amino acids from carboxyl terminus of angiotensin I to form the active octapeptide angiotensin II, which is an extremely powerful vasoconstrictor. Many synthetic drugs as Captopril, Benazepril, Lisinopril, Enalapril, ect. are widely used in the clinical practice. All of them have the same mechanism of action. In recent years studies have been mainly focused on the identification of effective peptides, able to inhibit ACE activity with the aim to control hypertension and prevent cardiovascular diseases. Most of peptides inhibitors of ACE are relatively short sequences containing from 2 to 12 amino acids. Among them di- and tripeptides are more favorable as potential functional food additives candidates due to their high antihypertensive activity and low bitterness. Fernandez et al. [3] emphasize the role of ACE inhibitor peptides as a target for drug design resulting from the function of ACE in cardiovascular and renal diseases.

The aim of this study is to synthesize a short peptides, as a novel inhibitors of angiotensin-I converting enzyme (ACE), which were predicted to possess better ACE inhibitory activity and lesser side effects and as well as potential candidate as additives in functional foods of hypertension.

EXPERIMENTAL

Synthesis of the peptides

The chemical synthesis of target short peptides H-Ile-Ala-Pro-OH, H-Leu-Ala-Pro-OH, H-Val-Ala-Pro-OH and H-Val-Ala-Trp-OH was realized by Fmoc– strategy of solid phase peptide synthesis (SPPS) or by microwave assisted SPPS, performed on Syro Wave Peptide Synthesizer from Biotage.The TBTU/DIPEA method was used for coupling of each amino acid. For the synthesis of the peptides with free C-terminal group 2-chlortrityl chloride resin (CLTR) and Wang resin were used by us successfully. On each step of the synthesis was checked for free amino group with acetaldehyde/chloranil test. The target peptides were synthesized in similar procedure.

The synthesis begins with the coupling of the first amino acid Fmoc-Ala-OH to the resin. To the pre-activated resin are added 4 equiv. Fmoc-Ala -OH (0,093 g, 0,30 mmol), 4 eq. coupling agent TBTU (0,9563 g, 0,30 mmol), 8 eq. base DIPEA (0,7621 g, 0,60 mmol), dissolved in 2 ml DMF. The reaction mixture was allowed to mix for 1 hour and 30 minutes at a Vortex mixer.

* To whom all correspondence should be sent:
E-mail: bkq_1982@abv.bg

Completion of coupling reactions were monitored by the Kaiser test and the Fmoc groups were removed by adding 20% piperidine in DMF. After completion of the reaction is carried acetaldehyde / chloranil test and in the case of is absence of a free -NH_2 group the reaction continue with the next step. Deblocking was performed with 20% piperidine solution. The second amino acid - Fmoc-Val-OH was added in the following way: to 2 ml DMF was added 4 equiv. Fmoc-Val -OH (0,105 g, 0,30 mmol), 4 eq. coupling agent TBTU (0,9563 g, 0,30 mmol) and 8 eq. base DIPEA (0,102 ml, 0,60 mmol). The mixture thus obtained was added to the resin in the reaction vessel. The reaction system was of a Vortex mixer for 1.5 h. After this Fmoc protection is accomplished by blocking with 20% solution of piperidine. The final step of the synthesis of the tripeptide is its removal from the resin. The most commonly used reagent for removal of the synthesized peptide from the resin is trifluoro acetic acid (TFA). During the reaction are formed highly reactive carbcations. This requires the use of quenchers that prevent adverse reactions. In our case we used water and triisopropyl silane (TIS). A solution consists of 95% TFA: 2,5% H_2O: 2,5% TIS, was added to the resin and reaction mixture was mixed for 3 h under magnetic stirring at room temperature. After completion of the reaction the flask contents were filtered and washed with TFA, evaporated under nitrogen and precipitate by diethyl ether under ice. The organic solvents were removed and the peptides were freeze drying.

Purification and characterization of target peptides

The obtained tripeptides were purified by HPLC and characterized by UPLC-MS and NMR analysis. ^{1}H NMR (600 MHz,DMSO-d_6, δ ppm):data for **H-Leu-Ala-Pro-OH:** 4.63 (1H, α -H-Ala), 4.23 (1H, α -H-Pro), 3.60, 3.41 (2H, δ -CH2-Pro), 3.21 (1H, α-Leu), 2.33, 1.94 (4H, CH_2-Pro), 1,79 (1H γ- CH-Leu) ,1.45, 1.38 (2H, β- CH_2-Leu), 1.41 (3H, CH3-Ala), 0,92 (6H, CH_3-Leu); **Ile-Ala-Pro-OH**: 4.76 (1H, α-H-Ala), 4.43 (1H, α-H-Pro), 4.03 (1H, α-H-Ile), 3.61, 3.74 (2H, δ-CH_2-Pro), 2.2 -1.84 (4H, CH_2-Pro), 1.96 (H, β-CH-Ile), 1.56 (3H, CH_3-Ala), 0,96 (3H, CH_3-Ile), 0.82 (3H, CH_3-Ile); **H-Val-Ala Pro-OH**: 4.56 (1H, α-H-Ala), 4.13 (1H, α-H-Pro), 4.06 (1H, α-H-Val), 3.61, 3.54 (2H, δ-CH_2-Pro), 2.2 -1.84 (4H, CH_2-Pro), 1.94 (H, β-CH-Val), 1.56 (3H, CH_3-Ala), 1.01 (3H, CH_3-Val), 0.92 (3H, CH_3-Val) ; **H- H-Val-Ala-Trp-OH**: 7,49-6.93 (4H Ar Trp), 7.10 (1H Trp) (1H4.90 (1H, α-H-Trp), 4.34 (1H, α-H-Ala), 3.76 (1H, α-H-Val), 3.31-3,01 (2H, β-CH_2-Trp), 1.82 (H, β-CH-Val), 1.50 (3H, CH_3-Ala), 0.92 (6H, CH_3-Val).

The analyses of the peptides were performed by UPLC connected with MS detector. The samples were applied to a column: ACQNITY HPLC BEH C18, 1,7 mm, isocratic gradient elution with mobile phase: CH_3CN/0.1 % TFA:H_2O/0.1 % TFA (30:70 v/v) and after that purified with High liquid performance chromatography - HPLC Agilent 1200, column : RP Synergi 4 Fusion, mobile phase AcCN:H_2O (30:70 v/v).

Determination of ACE inhibitory activity of the peptides

To determine ACE inhibitory activity was used modified method of *Jimsheena and Gowda* [4]. The analysis is based on interaction between ACE and the specific synthetic peptide hippuryl-histidyl-leucine (HHL) in borate buffer pH 8,3 at 37°C. The released hippuric acid (HA) when complexed with pyridine and benzene sulfonyl chloride (BSC) forms a yellow color, which is measured at 410 nm.

For each assay, a sample solution of ACE inhibitor (25µl) with 50 ml of 5 mM Hip-His-Leu in 125 µl 0,05 M sodium borate buffer with pH 8,3 containing 300 mM NaCl was preincubated at 37° C for 30 min. with 25µl ACE from rabbit lung, as obtained mixture was stirred at Vortex mixer for 5 min. The reaction was stopped by adding 100 µl 1N HCl, followed by added of freshly distilled 400 µl pyridine and 200 µl BSC at Vortex mixer for 1 min and cooled with ice bath. The absorbance was measured at 410 nm on Thermo Scientific Evolution 300 UV-VIS.

The extent of inhibition was calculated as follows:

ACE inhibitory activity (%) = [1 – (A/B)] x 100,

Where: A is the absorbance of the sample, containing enzyme, substrate and inhibitor , B is the absorbance of the sample, containing enzyme and substrate.

All measurements were performed in triplicate. A calibration curve was obtained by plotting the absorbance at 410 nm versus different hippuric acid (HA) concentrations. As reference was used Lizinopril, which inhibit 100 % ACE .

RESULTS AND DISCUSSION

The inhibitors of ACE are among the most prospective resources at therapy of hypertension, heart attack, diabetic hypertension as different cardiovascular diseases. Major efforts worldwide are directed towards synthesis of effective peptides

and peptidomimetics, as inhibitors of Angiotensin I-converting enzyme for the pharmacy and like supplements for functional foods. It is known that all experiments on peptide inhibitors of ACE are conducted with samples isolated from different natural sources. Biologically active peptides, which have been identified are contained in a number of natural and processed food products -milk, eggs, fish, soya, rice, spinach, different hydrolysates and etc. [5,6,7,8,9,10,11], whose activity results in the formation of versatile effects - immunomodulating, antibacterial, opioid, antithrombotic, antihypertensive (ACE inhibitors). The design of peptides for therapeutic purposes requires detailed information on conformational requirements and maximum biological activity.

Based on the relationship between structure-function of ACE inhibitors can be found some general similarities between them [12]. They are rich in hydrophobic amino acids on S'_2 subsite and many of them contain proline, lysine or arginine as C-terminal residues. The majority among ACE-peptide inhibitors are di- or tri- peptides that are resistant to endopeptidase of the digestive tract and can be readily absorbed into the blood [13]. The amounts of these peptides is insignificantly and vary in milligram amounts. Of interest is to investigate the inhibitory activity of the chemically synthesized peptide inhibitors of ACE.

The chosen approach with Fmoc-strategy in solid phase synthesis, proved particularly effective and yields of target peptides in this study were within 65-77% of the pure substance. Strategy using 2-Cl-trityl-chloride resin has proved particularly effective in the synthesis of the proline tripeptides. The reaction scheme of synthesis of the H-Val-Ala-Pro-OH is shown on Fig.1.

All synthesized peptides H-Ile-Ala-Pro-OH, H-Leu-Ala-Pro-OH, H-Val-Ala-Pro-OH and H-Val-Ala-Trp-OH were identified and characterized by ^{1}H NMR and UPLC. The UPLC chromatogram of the purified H-Val-Ala-Pro-OH are shown on Fig.2.

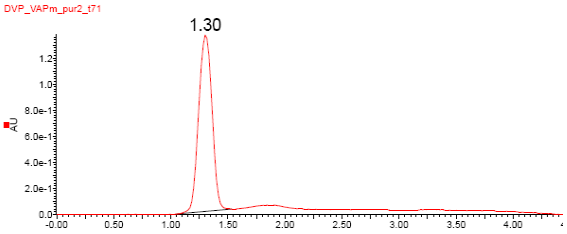

Fig.2. UPLC chromatogram of the purified peptide H-Val-Ala-Pro-OH

The activity of ACE inhibitors can be determined by various analytical methods. The most popular tests for determination of ACE inhibitory activity of peptides in vitro are based on spectrophotometric, fluorimetric, colorimetric and radiochemical methods and chromatographic techniques. The measurement of IC_{50} is a marker for biological activity, but not for antihypertensive action.

It is not established a clear relationship between ACE inhibitors and antihypertensive activity. It is possible the antihypertensive activity to exceed expectations or in some cases there is no antihypertensive activity. The most significant reasons for this are the oral bioavailability of studied peptides and mechanisms other than the inhibition of ACE.

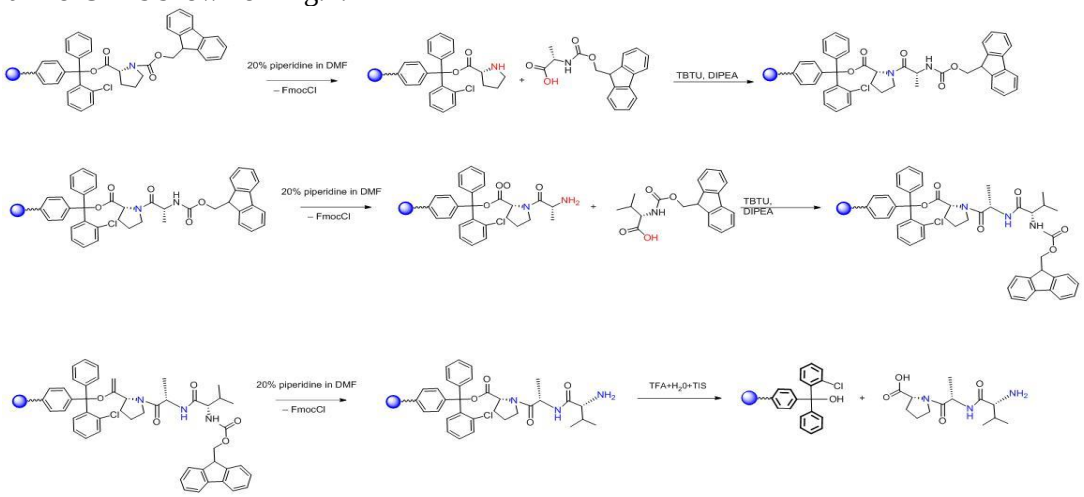

Fig.1. Reaction scheme of the synthesis of H-Val-Ala-Pro-OH

To determine the ACE inhibitory activity we used a modified colorimetric method of Jimsheena and Gowda. The analysis of the ACE inhibitory activity is based on the interaction of ACE with the

specific synthetic substrate HHL (hippuryl-L-histidyl-L-leucine) in phosphate buffer with pH 8.3 at 37 ° C, which leads to the release of hippuric acid and dipeptide histidil- L-leucine (HL).

The released hippuric acid forms a complex with pyridine and benzenesulfonyl chloride. On fig.3 is shown a possible mechanism of the reaction of hippuric acid with pyridine and benzenesulfonyl chloride.

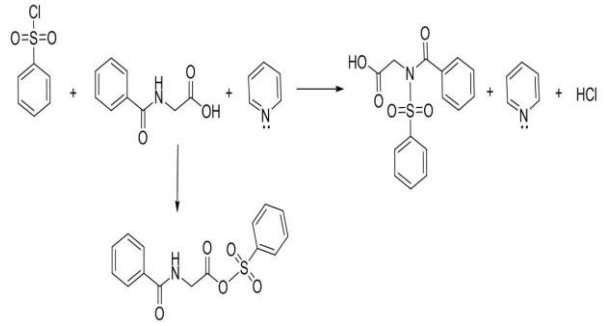

Fig. 3. A possible mechanism of interaction between hippuric acid and benzene sulfonyl chloride in the presence of pyridine

The complex of pyridine and BSC with hippuric acid gives yellow color and measured at 410nm. The color intensity is correlated with the concentration of hippuric acid. In the presence of ACE inhibitor in the reaction mixture the release of the product hippuric acid is inhibited, which can affect the absorbance values.

The determined relative inhibitory activity of synthesized peptides is shown on Fig.4.

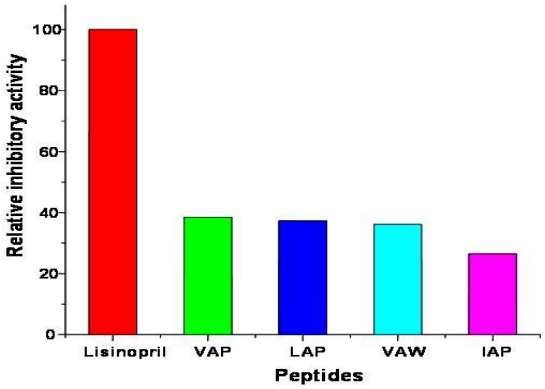

Fig. 4. Relative inhibitory potential of synthesized peptides

The figure shows that the VAP is with the highest inhibitory activity, and the lowest activity shows IAK.

The synthesized peptides, H-Ile-Ala-Pro-OH, H-Leu-Ala-Pro-OH, H-Val-Ala-Pro-OH and H-Val-Ala-Trp-OH be juxtapose with the commercially used Lisinopril. We hope they will be good additives in functional foods of hypertension.

CONCLUSION

Important role of RAS in cardiovascular system causes recently more and more attention. ACE inhibitors have always been used as the first line treatment of hypertension and heart failure. The design and synthesis of new synthetic structures as H-Ile-Ala-Pro-OH, H-Leu-Ala-Pro-OH, H-Val-Ala-Pro-OH and H-Val-Ala-Trp-OH that are good inhibitors of ACE is very promising for receiving a new drugs for heart diseases and functional foods for prevention of hypertension.

REFERENCES

1. M.A., Ondetti, D.W. Cushman, *Annu. Rev.Biochem.* **51**, 283 (1982).
2. H. Roks, Buikema, Y.M. Pinto, W.H. van Gilst, *Heart Vessels*, **12,** 119 (1997).
3. J.H. Fernandez, G. Neshich, A. Camargo, *Genet. Mol. Res*, **3**, 554 (2004).
4. V.K. .Jimsheena, L.R. Gowda, *Anal. Chem.* **81**, 9388 (2009).
5. G.-H. Li, H. Liu, Y.-H. Shi, G.W. Le, *J. Pharmaceut. Biomed. Anal.*, **37**, 219 (2005).
6. J.Tsai, J.-L., Chen, B.S. Pan, *Proc.Biochem.*, **43**, 743 (2008).
7. Y. Nakamura, N. Yamamoto, K. Sakai, T. Takano, *J. Dairy Sci.*, **78**, 6 (1995).
8. H., Wu, H.-L. He, X.-L., Chen, C.-Y., Sun, Y.-Z., Zhang, B.-C. Zhou *Proc.Biochem.*, **43,** 457 (2008).
9. A. Jang, M. Lee, *Meat Science*, **69**, 653 (2005).
10. D, Kitts, K. Weiler, *Current Pharmaceuti. Design*, **9,** 1309 (2003).
11. Y. Yang, E.D. Marczak, H. Usui, Y. Kawamura, M. Yoshikawa, *Journal of Agricultural and Food Chemistry*, **52**(8), 2223 (2004).
12. N. Yamamoto, *Biopolymers*, **43**, 129 (1997).
13. P. Sandeya, A text book of medicinal chemistry, Third edition, Elsevier, India, 2004, Chap. 27, 33.

МОЛЕКУЛЕН ДИЗАЙН И СИНТЕЗ НА ПЕПТИДНИ ИНХИБИТОРИ НА АНГИОТЕНЗИН I ПРЕВРЪЩАЩИЯ ЕНЗИМ ЗА ЛЕЧЕНИЕ И ПРЕВЕНЦИЯ НА ХИПЕРТОНИЯ

Б. Якимова, Д. Петкова И. Стойнева

Институт по Органична Химия с Център по Фитохимия, Българска Академия на Науките, ул. Акад.Г. Бончев, бл.9, 1113, София,България

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(Резюме)

Хипертонията и сърдечно-съдовите заболявания са едни от най-рисковите заболявания и представлават около 60% от общата статистика на заболяваемост у нас. С въвеждането на синтетичните инхибитори на ангиотензин превръщащия ензим (ACE) в клиничната практика настъпва нова ера в лечението на тези заболявания. Получените чрез твърдо фазен пептиден синтез нови късоверижни пептидни инхибитори на ACE - H-Ile-Ala-Pro-OH, H-Leu-Ala-Pro-OH, H-Val-Ala-Pro-OH и H-Val-Ala-Trp-OH бяха пречистени чрез HPLC и анализирани чрез UPLC-MS и ЯМР анализи. Те проявяват инхибиторна активност сравнима с тази на търговския препарат Lizinopril, което води до получаването на важна информация за различни синтетични структури като потенциални инхибитори на ACE, което дава възможност за молекулен дизайн и синтез на лекарствени форми с ясно изразен антихипертензивен ефект при пациенти страдащи от сърдечно-съдови заболявания.