ISOLATION AND CHARACTERIZATION OF PEPTIDES FROM MILK AS NATURAL INHIBITORS OF ACE I AND FOOD ADDITIVES

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Received 05 January 2024
Accepted 28 April 2024 *DOI: 10.59957/jctm.v59.i4.2024.5*

ABSTRACT

Inhibition of Angiotensin-converting enzyme I (ACE I) is a modern therapeutic approach to treatment of hypertension. In recent years, research into natural ACE peptide inhibitors without side effects has become important. The aim of this study is to isolate and characterize novel bioactive peptides from skim and/or whole cow's milk fermented with selected lactobacillus strains. Several homo/heterofermentative strains of the Lactobacillus species of dairy origin have been pre-selected and different milk fermented samples have been studied. A protocol for analyses was designed and the milk proteins were separated by centrifugation at 4°C at 10000 × g, with molecular mass cut off (MWCO) membranes of 3 and 10 kDa. The samples with molecular mass below 3 kDa were further separated by ultrafiltration by dialysis cell (cut off membrane 1 kDa) by continuous stirring at room temperature. The milk fractions under 1 kDa molecular mass were characterized by UPLC-MS. The ACE-inhibitory activity was determined using the FAPGG (N-[3-(2-Furyl) acryloyl]-L-phenylalanyl-glycyl-glycine) degradation method. All tested samples (1 kDa) exhibit ACE I inhibitory activity with IC_{50} in a range of 6 - 37 mg mL^{-1}. In silico logP prediction of selected peptides was used to assess whether lipophilicity of the compounds falls within the so-called "therapeutically relevant pharmacokinetic space".

Keywords: Angiotensin-converting enzyme, ACE I, Lactobacillus, milk peptide, inhibitor, lipophilicity.

INTRODUCTION

Angiotensin-converting enzymes (ACE I) I and II are multifunctional enzymes produced by many cells in the body. The different peptide inhibitors of ACE I are important targets in the treatment of hypertension and other cardiovascular diseases. Presently, the development of new natural peptides with antihypertensive effect has attracted much attention in order to minimize side effects of the traditional drugs used for the treatment of hypertension.

Peptides with ACE I inhibiting properties have been identified from various sources, including cheese [1], eggs [2], kefir [3] fish [4], soy [5], peas [6], sour

milk [7], and yogurt [8]. Short peptides derived from milk and different milk products have proven to be very effective inhibitors of ACE I. They can be formed from the two main types of proteins found in milk: caseins (α-, β- and κ-caseins) and whey proteins, for example, α-lactalbumin and β-lactoglobulin. A promising approach is to obtain bioactive peptides from alternative sources, such as milk and milk products fermented with new lactic acid bacteria (LAB) strains. The obtained whey proteins and polypeptides display high proteolytic activity and can become promising and valuable tools for obtaining new natural peptides with specific bioactivities at a low production cost. Mention deserves the fact that fermented dairy products produced with *Lactobacillus delbrueckii, Lactobacillus helveticus, Lactobacillus delbrueckii subsp. bulgaricus,* and *Streptococcus salivarius subsp. thermophilus* are rich in bioactive peptides. These peptides have the potential to contribute to the control of hypertension [9,10]. The intricate interplay of these specific strains in the fermentation process seems to yield not only delicious dairy products but ones with promising health benefits. Xia et al. reported that Gly-Ala (GA) peptide isolated from whey proteins after fermentation with *Lactobacillus plantarum* QS670 could be used as a functional food in preventing or reducing hypertension-associated diseases [11].

In silico modeling approaches are widely used for pre-screening of substances with potential medicinal properties, due to optimality and cost-effectiveness of computational techniques. They are used in various aspects of drug discovery and development, such as drug design and pharmacokinetic and pharmacodynamic analyses. Oral delivery of drugs, nutritional supplements as well as other active substances faces a number of challenges related to low water solubility and/or low ability to pass through the intestinal cell membrane. Solubility of hydrophobic active substances is one of the two main factors determining their bioavailability. The octanol-water partition coefficient (logP) of substances is an easily determined indicator of the propensity of a neutral, uncharged, compound to dissolve in an immiscible two-phase system of lipids (fats, oils, organic solvents) and water. Lipophilicity is usually expressed as the logarithm of the partition coefficient. A negative value for logP means that the compound has a higher affinity for the aqueous phase (hydrophilic character). When logP = 0 the compound is evenly distributed between the lipid and aqueous phases; a positive value for logP means a higher concentration in the lipid phase (i.e., the compound is lipophilic). LogP = 1 means that there is a 10 : 1 distribution in the organic:aqueous phases. Although there are well-established experimental methods for measuring the distribution coefficient, they can be laborious and expensive. Prediction of logP even before a substance is synthesized offers a unique means of guidance for scientists. For drug molecules and drug candidates, the logP value indicates where they will be distributed: hydrophilic substances will be dissolved in the blood and lipophilic substances in the lipophilic membranes of cells. According to Lipinski's Rule of 5, the logP of a compound intended for oral administration should be < 5. The values between logP = 3 and logP = 5 are considered optimal for drug candidates.

The aim of the present study is isolation and characterization of short bioactive peptides as inhibitors of ACE I from whey proteins of milk fermented with several strains of LAB. *In silico* lipophilicity predictions based on molecular structures have been also made in order to assess whether lipophilicity of the compounds falls within the so-called "therapeutically relevant pharmacokinetic space".

EXPERIMENTAL

Materials and Methods

Enzymes and reagents

Angiotensin I-converting enzyme from rabbit lung and N-[3-(2-Furyl) acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) were purchased from Sigma Aldrich. Lisinopril (LIS), (N^2-[-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline) was obtained from SuanPharma (Spain).

Microorganisms, culture conditions and milk fermentation

In the present work, several LAB strains from the collection of LAB & Probiotics Laboratory at the Stephan Angeloff Institute of Microbiology, BAS, were used. They were isolated from traditional home-made fermented products from different regions of Bulgaria, or were of human origin. Selected LABs were applied as base for starter/adjuncts used as single of a multi-species formula, as follows: (i) a *Lactobacillus helveticus*

strain NS1 (from artisanal yogurt made in a small farm in the Central Balkan) was used as a single starter and mixed multibacterial formula from lactobacilli, with a variety of species and origin; (ii) a mix of 5 strains belonging to the species *Lactiplantibacillus plantarum* NS 25 and AC 11S *(*from home-made white brined cheese), *Limosilactobacillus fermentum* LF3 and *Lacticaseibacillus rhamnosus* 2HS (of human origin) and mixed numerous cultures (MILKs all): 14 *Lactiplantibacillus plantarum* strains from katak (L1-L14) one strain of *Lactobacillus delbrueckii bulgaricus* Lbb 51S from sheep yogurt + *L. plantarum* AC131, and two strains of *Limosilactobacillus fermentum* (vaginal samples) [12, 13]. All samples were stored at -20°C in MRS (Merck, Darmstad, Germany) broth supplemented with 20 % v/v sterile glycerol (Merck). Prior to the experiments, the strains were pre-cultured twice in MRS broth. The cells from exponential cultures were harvested by 5 min centrifugation at 9000 × g (Hermle, Germany) and washed with sterile PBS pH 6.0. They were inoculated in reconstituted (12 % w/v) skim (Serva, and PicCo LTD, Bulgaria), or whole (3.6 and 2 % fat) cow milk sterilized at 100°C for 10 min, and used as starters (5 - 10 % v/v). Fermentation with multi-bacterial formula of five strains was obtained with a mixed inoculum 10 % v/v, in equal proportions of all LAB cultures in corresponding milks. Milk fermentations from 4 h to 24 h were carried out at 37°C in Nuve EN 400 (Turkiye) incubator. Fermented milks were stored at 4 - 10°C in the refrigerator room for different periods before the peptide fractions analyses. All 17 milks were fermented in whole cow milk (3.6 % fat) as pure cultures and, after the end of fermentation, the mix of all fermented products was stored for one month at 4°C.

The obtained whey fractions by simple filtering (sterile paper filters) or by centrifugation at 6000 × g (10 min) were subjected to analysis for bioactive peptides.

Isolation of milk peptides by ultrafiltration

The milk protein samples were separated by centrifugation at 4°C at 10000 × g for 20 min, with molecular mass cut-off (MWCO, Amicon Ultra 15 mL, Millipore, Billerica, MA) membranes of 3 and 10 kDa to collect peptide fractions (< 3 kDa, > 3 kDa, > 10 kDa, < 10 kDa, 3 - 10 kDa). The samples with molecular mass below 3 kDa were further separated by ultrafiltration by dialysis cell (cut-off membrane 1 kDa) by continuous stirring at room temperature. The collected samples were lyophilized and kept at -20°C for further characterization.

Determination of soluble protein content

Protein content in the milk samples was determined by the Bradford method, which involves binding of Coomassie Brilliant Blue G-250 to protein in the sample [14]. 20 µL of each sample (2 mg mL^{-1} in PBS buffer, pH 7.2) was mixed with 1 mL of Bradford reagent and then incubated for 5 min. Absorbance of the sample was measured at 595 nm in multiplate reader Clariostar Plus (BMG Labtech, Software: Smart control V6.20). The protein content of the samples was calculated using a standard curve of bovine serum albumin 2 mg mL^{-1}, stock solution, Bio-Rad), with dilutions from 0.125 mg mL^{-1} to 1 mg mL^{-1}.

Peptide identification by LC-HRMS analysis

Peptide separation was carried out with a Q Exactive® Hybrid Quadrupole-Orbitrap® Mass Spectrometer (ThermoScientific Co, USA) equipped with a HESI® (heated electrospray ionization) module, TurboFlow® Ultra High Performance Liquid Chromatography (UHPLC) system (ThermoScientific Co, USA) and HTC PAL® autosampler (CTC Analytics, Switzerland).

Evaluation of ACE inhibitory activity and IC_{50} value

The FAPGG degradation method of Holmquist [15], with substantial modifications as described in our previous work, was used [16]. In brief, the assay was conducted as ACE (250 mU) was dissolved in 1 mL TRIS buffer (0.075 M without NaCl, pH 8.3) and kept at -20°C. A working solution 1 mM of FAPGG in the assay buffer was prepared and kept stable for two months in a brown glass bottle at 4°C. Subsequent dilutions of the tested compounds were done in an assay buffer (TRIS buffer 0.075 M with 0.3 M NaCl, pH 8.3).

In short, the experimental procedure was as follows: 10 µL of ACE (2.5 mU) plus 10 µL buffer, or 10 µL of inhibitor solution, were mixed with 230 µL assay buffer in plastic tubes and the mixture was incubated at 37°C for 5 min. The reaction started with 0.75 mL of 37°C preheated solution of FAPGG substrate (0.75 mM in assay buffer final concentration). After a gentle mix of the tubes, FAPGG degradation was followed up at 340 nm

for 30 min with Carry double-beam spectrophotometer, with buffer as reference. ACE activity was expressed as a rate of disappearance of FAPGG ($\Delta A\ min^{-1}$). A Tris-HCl buffer was used instead of peptide solutions in the control experiment. The ACE inhibition rate (I) was calculated by means of the following equation:

$$I\ (\%) = \frac{A_{blank1} - A_{sample}}{A_{blank1} - A_{blank2}} \times 100$$

where A_{blank1} was absorbance without sample, A_{blank2} was absorbance without enzyme working solution, and A_{sample} was absorbance with samples or positive control.

In silico logP prediction

Hyperchem software was utilized to optimize the geometrical structure of the peptide sequences using the semi-empirical Parametric Method 3 (PM3) for calculating the molecular electronic structure and, subsequently, for evaluating their lipophilicity [17].

RESULTS AND DISCUSSION

Microorganisms, culture conditions and milk fermentation

Fermentation of Bulgarian yoghurt with a symbiont starter of *Lactobacillus delbrueckii bulgaricus* and *Streptococcus salivarius subsp.* usually takes place in the course of 4 h, at 42°C. According to the Bulgarian State Standard (No 1210 in 2010), it has been established that Bulgarian starters form a good coagulum within that time, with very little whey, preserving the specific properties of the traditional Bulgarian yoghurt. For the present work, however, strains with less pronounced proteolytic activity were selected, and with improved metabolic conversion abilities as compared to those in the starter. Heterofermentative lactobacilli were optionally added, which, along with lactic acid in the fermentation of glucose and proteins, also produce acetic acid, propionic acid and various low-molecular-weight-active products.

All strains were from the collection of LAB & Probiotics Laboratory of the Stephan Angeloff Institute of Microbiology, BAS, and some of them have been shown to have high biological activity, e.g., suppression of pathogens and fungi development due to the production of various active peptides and short-chain fatty acids [18].

Our selected milk samples included both some fermented with single strains of lactobacilli (e.g., *Lactobacillus helveticus NS1* only), and some fermented with a combination of different *Lactobacilli* strains able to induce proteolysis in milk proteins in a different way.

The sample fermented only with *Lactobacillus helveticus* NS1 was stored for a long period in order to evaluate the entire spectrum of short-chain peptides obtained by expected ACE I inhibitory activity resulting from natural proteolysis, without addition of proteolytic enzymes (trypsin, pepsin, etc.).

The analysis of literature data has shown that samples with molecular mass below 1 kDa obtained from different sources (skimmed cow's milk, beef milk, different types of cheese, etc.) most often exhibit the highest ACE I inhibitory activity [19, 20, 21]. From the numerous experiments that were carried out, four different milk samples fermented with different strains of LAB were selected for detailed study. Summary information about the studied samples is presented in Table 1.

ACE I inhibitory activity of the studied peptide fractions of milk with different molecular mass was measured at five different concentrations, and all measurements were made in triplicate under identical conditions. For inhibitor control, a proven and widely used inhibitor Lisinopril was also used in five different concentrations. ACE inhibitory activities have been defined as IC_{50} values, because peptide concentration requires reduction of the ACE activity by half. The results of lyophilized samples with molecular mass below 1 kDa are presented in Table 2. Analysis of the results has shown that the peptides in LB sample exhibit the highest IC_{50} value: 6.1 mg mL^{-1}. These results are in good agreement with those obtained for the well-known antihypertensive peptide Ile-Pro-Pro 1.3 mg mL^{-1} isolated from milk κ-casein [7]. ACE I has two active sites: one in the C-domain and another in the N-domain. ACE C-domain is considered a promising pharmacological target for reducing blood pressure. Hydrophobic and positively charged amino acids, along with Pro in the C-terminal position, have been assumed as contributing to better affinity of the peptides for ACE [22].

Quantitative analysis of milk samples

HPLC-MS/MS was employed to analyze

Table 1. Selected milk samples for further investigation: fermentation, used strain and origin.

Milk sample, abbreviation	Fermentation	Strain	Origin
Sample 1, LH	12 % skim milk (Scharlau, Spain)	Pure culture *Lactobacillus helveticus* NS1	New isolate from artisanal yogurt
Sample 2, All strains (AS)	commercial whole cow milk	20 strains *Lactobacillus sp.* with diary and human origin	From the collection of Laboratory of "LAB & Probiotics", the Stephan Angeloff Institute of Microbiology
Sample 3, LB	L (-) cow milk	*Lactobacillus bulgaricus limousine*, 2 strains *Lactobacillus fermentum* and *Lactiplantibacillus plantarum* from cheese	From the collection of Laboratory of "LAB & Probiotics", the Stephan Angeloff Institute of Microbiology
Sample 4, MIX A-KR 51 (KR 51)	commercial whole cow milk	*Lactobacillus brevis* (katak), *Lactobacillus gasseri* and two strains *Lactiplantibacillus plantarum*	From the collection of Laboratory of "LAB & Probiotics", the Stephan Angeloff Institute of Microbiology

Table 2. The determinated IC_{50} values for peptide fractions with molecular mass below 1 kDa.

Sample	IC_{50}
LB	6.1 mg mL^{-1}
All strains	9.2 mg mL^{-1}
LH	12.1 mg mL^{-1}
MIX A-KR 51	37.0 mg mL^{-1}

quantitatively the milk fractions with molecular mass below 1 kDa. Chromatographic separation was carried out in a Nucleoshell C18 (100 × 2.1 mm, 2.6 µm) analytical column (Macherey-Nagel, Germany), with gradient elution at 300 µl/min flow rate. Samples were loaded into a C_{18} column after initial washing with buffer A (0.1 % (v/v) formic acid in water). Peptides were eluted over 10 min with 0-40 % buffer B (0.1 % (v/v) formic acid in acetonitrile), followed by 40-90 % buffer B over 3 min. The column eluate was directed into a HESI source of the mass spectrometer. The Q-executive parameters were: spray voltage 4.0 kV, sheath gas flow rate 32, auxiliary gas flow rate 10, capillary temperature 320°C, probe heater temperature 300°C, and S-lens RF level 50.

Full-scan spectra over the 200 - 1200 m/z range were acquired in the positive ion mode at resolution settings of 70 000. All MS parameters were optimized for sensitivity target analyses using instrument control software. Tandem MS was performed for the top five most intense precursor ions selected for HCD fragmentation, with 30 % normalization collision energy and using settings of 17 500 resolutions and 4.0 amu isolation window with dynamic exclusion for 30 s. Ions with charge 4+ and above were excluded. Data acquisition and processing were carried out with Xcalibur 2.4® software package (Thermo Scientific Co, USA).

In recent years, there has been a rising interest in the fermented milk products as a natural source of ACE inhibitor peptides produced during hydrolysis of the milk protein by microbial proteases presented in the starter culture. The results in this study obtained from the mass spectrometric analysis for fractions with a molecular mass below 1 kDa show the most characteristic peptide sequences occurring in the investigated samples: they are presented in Table 3.

Many literature data have proved that positively

Table 3. Peptide sequences of milk samples identified by UPLC-MS/MS analysis.

Milk sample, abbreviation	Peptide Sequence	MW
LH	Pro-Pro-Lys	341.22
	Leu-Thr-Arg	389.25
	Leu-Pro-Pro-Lys	454.39
	Met-Ala-Pro-Lys	446.24
All strains (AS)	Leu-Leu-Arg	401.28
	Leu-Phe-Arg	435.27
	Leu-Leu-Phe-Arg	548.36
LB	Leu-Phe-Arg	435.27
	Leu-Leu-Phe-Arg	548.36

charged amino acids at the C-terminus, such as arginine (Arg), strongly influence the ACE inhibitory activity of peptides. Toopcham et al. have found that the presence of C - terminal Arg mainly affects competitive binding with the active site of the enzyme [23]. When C-terminal Arg is removed, a significant reduction in the inhibitory effect of the peptides has been observed. The results obtained in our study for characteristic peptides in the LB and All strains samples show that their good inhibitory activity is probably due to the presence of C-terminal Arg in the peptide sequence.

Some preliminary theoretical calculations were conducted to clarify the role of the terminal amino acid in the biological activity of the peptide sequences commonly found in fractions obtained from dairy products fermented with LAB (LPPK, PPK, LPK, LFP). These calculations were prompted by the results of earlier studies into the best IC_{50} values for synthetic short peptides, such as Leu-Ala-Pro and Leu-Lys-Pro, with terminal proline in the peptide sequence [16]. In the present study, the geometry of six structures, including Leu-Ala-Pro and Leu-Lys-Pro, with terminal proline in the peptide sequence, were optimized using Hyperchem software. The method used for optimization of the geometry of molecules and for evaluation of their lipophilicity is PM3, or Parametric Method 3. Fig. 1 shows the optimized structures and the corresponding logP values.

The logP values for the investigated peptides have varied between 1.95 and 5.03. Obviously, presence of the polar Lys amino acid increases the hydrophilic character of peptides. Most favorable has been the presence of phenylalanine: the logP value then exceeded 5. Leucine residue at the N-terminus has also been critical for keeping logP within the optimal range,

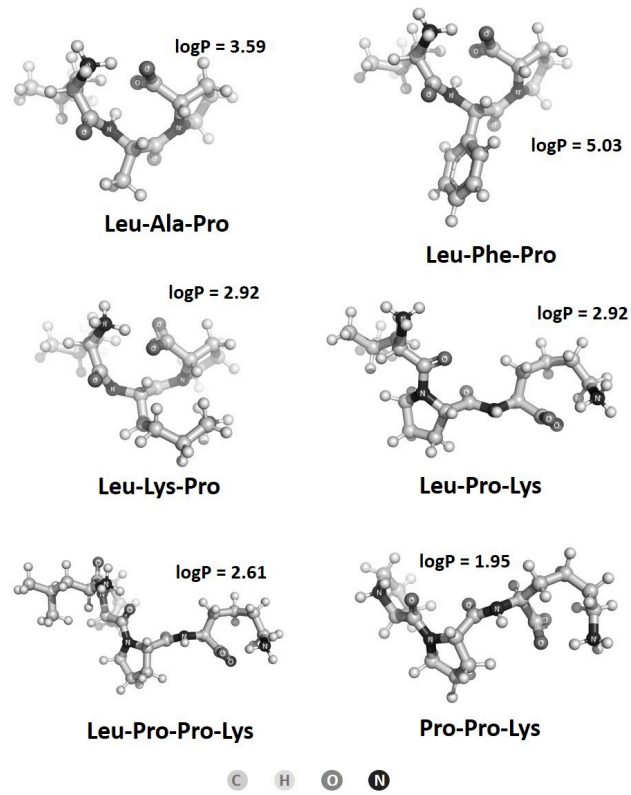

Fig. 1. The optimized structures of Leu-Ala-Pro, Leu-Phe-Pro, Leu-Lys-Pro, Leu-Pro-Lys, Leu-Pro-Pro-Lys, and Pro-Pro-Lys. The predicted logP values are also shown.

for its replacement with proline lowered the value by 1. Although logP is not the sole determinant of drug transport, formulation, dosage, clearance, and toxicity, it plays a critical role in determining the suitability of new drug/active agent candidates. It has been generally accepted that there is a "Goldilocks zone" between logP = 3 and logP = 5. The values predicted for Leu-Ala-Pro and Leu-Lys-Pro (peptides with the best IC_{50} values)

are close to optimal and warrant further theoretical studies with computational chemistry methods [16]. The values for LPPK and PPK (found in the LH sample) are technically outside the range, but the experimental IC_{50} show viable activity, which means that, although not being optimally delivered to the active site, they still have significant influence as ACE inhibitors that can be improved further with a proper delivery system.

CONCLUSIONS

In the present paper, production of natural short-chain peptides has been identified by employing selected lactic acid bacteria (LAB) strains and their proteolytic enzymes in milk fermentation. The results indicated presence of different peptides with ACE inhibitory activity.

From the logP values between 1.95 and 5.03 gained for the peptide sequences common in fractions obtained from dairy products fermented with LAB (LPPK, PPK, LPK, LFP), it can be concluded that the amino acid lysine increases the hydrophilicity of peptides. These results provide an insight into the lipophilicity of biopeptides, crucial for understanding their behavior in biological systems.

The obtained experimental and computational results supply valuable information on the ACE inhibitory activity of synthetic peptides and peptides derived from whey/milk protein hydrolysates. This knowledge may further contribute to the development of functional foods or therapeutics for management of hypertension.

Acknowledgements

The authors are grateful for the support of the Bulgarian Scientific Fund under Project CP-06-N 21/5, 2018. B.Y. and S.A. gratefully acknowledge the funding by the European Union-Next Generation EU, through the National Recovery and Resilience Plan of the Republic of Bulgaria, project № BG-RRP-2.004-0002, "BiOrgaMCT".

REFERENCES

1. J. Gómez-Ruiz, M. Ramos, I. Recio, Identification of novel angiotensin-converting enzyme-inhibitory peptides from ovine milk proteins by CE-MS and chromatographic techniques, Electrophoresis, 28, 2007, 4202-4211.
2. K. Majumder, S. Chakrabarti, J. Morton, S. Panahi, S. Kaufman, S. Davidge, J. Wu, Egg-derived ACE-inhibitory peptides IQW and LKP reduce blood pressure in spontaneously hypertensive rats, J. Funct. Foods, 13, 2015, 50-60.
3. A. Quirós, M. Ramos, B. Muguerza, M. Delgado, M. Miguel, A. Aleixandre, I. Recio, Identification of novel antihypertensive peptides in milk fermented with Enterococcus faecalis, Int. Dairy J., 17, 2007, 33-41.
4. Z. Qian, J. Je, S. Kim, Antihypertensive effect of anglotensin I converting enzyme-inhibitory peptide from hydrolysates of bigeye tuna dark muscle, Thunnus obesus, J. Agric. Food Chem., 55, 2007, 8398-8403.
5. A. Gurung, M. Ali, J. Lee, M. Abul Farah, K.M. Al-Anazi, Molecular docking and dynamics simulation study of bioactive compounds from Ficus carica L. with important anticancer drug targets, PLoS ONE, 16, 2021, e0254035.
6. R. Aluko, Determination of nutritional and bioactive properties of peptides in enzymatic pea, chickpea, and mung bean protein hydrolysates, J. AOAC Int., 91, 2008, 947-956.
7. Y. Nakamura, N. Yamamoto, K. Sakai, A. Okubo, S. Yamazaki, T. Takano, Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk, Journal of Dairy Science, 78, 1995, 777-783.
8. O. Kajimoto, T. Kurosaki, J. Mizutani, N. Ikeda, K. Kaneko, K. Aihara, Y. Nakamura, Antihypertensive effects of liquid yogurts containing "lactotripeptides (VPP, IPP)" in mild hyper tensive subjects, J. Nutr. Food, 5, 2002, 55-66.
9. S. Esmaeili, M. Mahmoudi, Z. Rezaieyazdi, M. Sahebari, N. Tabasi, A. Sahebkar, M. Rastin, Generation of tolerogenic dendritic cells using Lactobacillus rhamnosus and Lactobacillus delbrueckii as tolerogenic probiotics, J. Cell. Biochem., 119, 2018, 7865-7872.
10. Y. Usui, Y. Kimura, T. Satoh, N. Takemura, Y. Ouchi, H. Ohmiya, K. Kobayashi, H. Suzuki, S. Koyama, S. Hagiwara, H. Tanaka, S. Imoto, G. Eberl, Y. Asami, K. Fujimoto, S. Uematsu, Effects of long-term intake of a yogurt fermented with Lactobacillus delbrueckii ssp. bulgaricus 2038 and Streptococcus thermophilus 1131 on mice, Int. Immunol., 30, 2018, 319-331.

11. Y. Xia, J. Yu, W. Xu, Q. Shuang, Purification and characterization of angiotensin I-converting enzyme inhibitory peptides isolated from whey proteins of milk fermented with Lactobacillus plantarum QS670, Journal of Dairy Science, 103, 6, 2020, 4919-4928.
12. L. Dobreva, D. Borisova, T. Paunova-Krasteva, P. Dimitrova, V. Hubenov, N. Atanasova, I. Ivanov, S. Danova, From Traditional Dairy Product "Katak" to Beneficial Lactiplantibacillus plantarum Strains, Microorganisms, 11, 12, 2023, art. no. 2847.
13. S. Danova, D. Yankov, L. Dobreva, A. Dobreva, N. Armenova, A. Apostolov, M. Mileva, Postbiotics Production of Candidate-Probiotic Lactiplantibacillus plantarum AC131 with Renewable Bio Resources, Life, 13, 3, 2023.
14. M. Bradford. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal. Biochem. 72, 1-2, 1976, 248-54.
15. B. Holmquist, P. Bunning, JF Riordan, A continuous spectrophotometric assay for angiotensin converting enzyme, Anal. Biochem., 95, 2, 1979, 540-548.
16. B. Yakimova, P. Mateeva, P. Kardaleva, I. Stoineva, P. Todorova, R. Zamfirova, S. Yanev, *In vitro* and *ex vivo* studies on angiotensin-I converting enzyme (ACE), inhibitory activity of short synthetic peptides, Farmacia, 69, 2, 2021, 307-313.
17. J. Stewart, Optimization of Parameters for Semiempirical Methods II. Applications, J. Comput. Chem., 10, 1989, 221-264.
18. M. Yakovlieva, T. Tacheva, S. Mihaylova, R. Tropcheva, K. Trifonova, A. Tolekova, S. Danova, T. Vlaykova, Influence of Lactobacillus brevis 15 and Lactobacillus plantarum 13 on blood glucose and body weight in rats after high-fructose diet, Beneficial Microbes, 6, 4, 2015, 505-512.
19. J. Li, J. Zhao, X. Wang, A. Qayum, M. Hussain, G. Liang, J. Hou, Z. Jiang, A. Li, Novel Angiotensin-Converting Enzyme-Inhibitory Peptides From Fermented Bovine Milk Started by Lactobacillus helveticus KLDS.31 and Lactobacillus casei KLDS.105: Purification, Identification, and Interaction Mechanisms, Front. Microbiol., 10, 2019, art. no. 2643
20. B. Qian, M. Xing, L. Cui, Y. Deng, Y. Xu, M. Huang, S. Zhang, Antioxidant, antihypertensive, and immunomodulatory activities of peptide fractions from fermented skim milk with Lactobacillus delbrueckii ssp. bulgaricus LB340, J. Dairy Res., 78, 1, 2011, 72-79.
21. N. Estevez, C. Fucinos, A. Rodriguez-Sanz, M. Rua, Development and sensory test of a dairy product with ACE inhibitory and antioxidant peptides produced at a pilot plant scale, Food Chemistry, 394, 2022, art. no. 133459.
22. A. Rai, K, Sanjukta, K. .Jeyaram, Production of angiotensin I converting enzyme inhibitory (ACE-I) peptides during milk fermentation and their role in reducing hypertension, Critical Reviews in Food Science and Nutrition, 57, 13, 2017, 2789-2800.
23. T. Toopcham, J. Mes, H. Wichers, S. Roytrakul, J. Yongsawatdigul, Bioavailability of angiotensin I-converting enzyme (ACE) inhibitory peptides derived from Virgibacillus halodenitrificans SK1-3-7 proteinases hydrolyzed tilapia muscle proteins, Food Chem., 2017, 190-197.