Food Science and Applied Biotechnology, 2023, **6**(2), 395-404

Food Science and Applied Biotechnology
e-ISSN: 2603-3380

Journal home page: www.ijfsab.com
https://doi.org/10.30721/fsab2023.v6.i2



OPEN ACCESS

Research Article

The ACE-inhibitory activity of alcalase, papain and pepsin lupin protein hydrolysates

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Abstract

Recently, *Lupinus angustifolius* proteins revealed their high potential to liberate bioactive peptides after hydrolysis. In our study, we examined the release of angiotensin I-converting enzyme inhibitory peptides from extracted lupin proteins after digestion by alcalase, papain, and pepsin. Three enzyme-to-substrate ratios were evaluated with three hydrolysis duration times. First, the degree of hydrolysis was determined using the ortho-phthalaldehyde aldehyde (OPA) method and then, the HPLC-DAD method was applied to measure the ACE-inhibitory activity. According to our results, all hydrolysates possessed ACE inhibitory properties. The calculated IC_{50} concentrations varied from 0.26 ± 0.59 to 2.19 ± 0.23 $\mu g.ml^{-1}$. The peptides with the lowest IC_{50} values were produced by pepsin ($\Delta IC_{50} = 0.33 \pm 0.40$ $\mu g.ml^{-1}$), followed by papain ($\Delta IC_{50} = 0.67 \pm 0.61$ $\mu g.ml^{-1}$), and alcalase ($\Delta IC_{50} = 1.78 \pm 0.92$ $\mu g.ml^{-1}$). Further research on bioavailability is required to demonstrate the beneficial effects of bioactive peptides on human health, since they have to resist digestive processing pass the intestinal barrier, and reach the bloodstream and intended organs.

Keywords

protein hydrolysates, ACE, antihypertensive peptides, degree of hydrolysis, plant proteins

Abbreviations

ACE – angiotensin-converting enzyme; BSA – bovine serum albumin; CVD – cardiovascular diseases; DH – degree of hydrolysis; DTT – 1,4-dithiothreitol; HHL– hippuryl-histidyl-leucine; HPLC – high-performance liquid chromatography; NaOH – sodium hydroxide; OPA – O-phthaldialdehyde; PBS – phosphate-buffered saline

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Article history:
Received 25 May 2023
Reviewed 29 September 2023
Accepted 5 October 2023
Available on-line 11 October 2023

https://doi.org/10.30721/fsab2023.v6.i2.287
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Introduction

In recent years attention to bioactive peptides increases due to their possibility to influence different biological activities and to improve human health. They can be isolated from various sources: plants, animals, mushrooms, fruits, marine organisms and others (Zaky et al. 2022). To exert their function, first, they have to be liberated from the parent protein. The most popular techniques are *in vitro* hydrolysis by proteolytic enzymes or proteolysis by digestive enzymes (gastrointestinal digestion) (Rizzello et al. 2016). Bioactive peptides typically have amino acid residues ranging from 2 to 20, but sometimes their size is larger (Shahidi and Zhong (2008). Different types of biological activities are demonstrated such as antioxidant, opioid, antidiabetic, anti-inflammatory, hypocholesterolemic, antihypertensive, osteoprotective, and antitumor (Rizzello et al. 2016). Their integration in different food supplements, health-promoting foods and medicinal products is gaining more popularity. The study of their structure, functions and potential will be useful for their faster implementation.

Nutrition plays a key role in the prevention of cardiovascular diseases (CVD), such as atherosclerosis, coronary heart disease, stroke, and heart failure (Gouda et al. 2006; Iwaniak Minkiewicz and Darewicz 2014) According to the World Health Organization (WHO), these diseases are the leading cause of death worldwide (Daskaya-Dikmen et al. 2017). One of the most prevalent chronic diseases, hypertension (the continuous rise in blood pressure), is becoming a serious problem. Statistics show that over 20% of adults in the world are diagnosed with this condition. Lifestyle changes and diet are two of the main factors that contribute to lowering lower blood pressure (Vermeirssen et al. 2004). It has been shown that even small decreases lead to significantly lower CVD risks (Vermeirssen et al. 2004).

Various biochemical systems play a key role in the regulation of blood pressure, but the most important factor is the renin-angiotensin system, through which the angiotensin-converting enzyme (ACE) is inhibited. ACE (EC 3.4.15.1) plays a key role in controlling blood flow, pressure and electrolyte balance Mehanna and Dowling (1999). It increases blood pressure after the degradation of bradykinin and catalyses the conversion of angiotensin I to angiotensin II (Chauhan and Kanwar (2019). Blood pressure is lowered as a result of this enzyme's (ACE's) inhibition, contributing to the treatment of hypertension. The enzyme is a glycoprotein consisting of a carbohydrate part composed of N-acetylglucosamine, mannose, galactose, fructose, and N-acetylneuraminic acid Murray and FitzGerald (2007). In clinical practice, different medicaments have been extensively used as inhibitors: captopril, enalapril, ramipril, and trandolapril, but all of them have been reported to exert adverse effects such as coughing, taste abnormalities, and skin rashes (Lahogue et al. 2010). Suitable peptide sequences are considered a safer alternative. Depending on the chain length, amino acid composition, and sequence, the ACE-inhibitory action could vary. It is demonstrated that peptides with a stronger ability to bind the enzyme have an amino acid sequence of 2-12 amino acids. (Daskaya-Dikmen et al. 2017). They can inhibit the enzyme both competitively and non-competitively but in most cases, ACE prefers substrates or competitive inhibitors with hydrophobic (aromatic or branched side chain) amino acid residues at the three C-terminal positions (Daskaya-Dikmen et al. 2017). ACE inhibitors can only block one of the two catalytic sites of ACE due to differences in substrate selectivity between the two sites (Udenigwe and Mohan, 2014). However, more research is required to fully understand the precise inhibitory mechanism.

The isolation of ACE-inhibitory peptides from various food sources has been the subject of numerous studies (Muthia, Suganda and Sukandar no date; Barbana and Boye 2010; Pinto et al. 2012; He, Liu and Ma 2013). Unlike synthetic ACE inhibitors, ACE-inhibitory peptides derived from food resources are promising alternatives due to their bioavailability and absence of side effects (Daskaya-Dikmen et al. 2017). Such types of peptides are reported in various meat and milk products, marine organisms, different plants, cereals, mushrooms, etc. (Muthia, Suganda and Sukandar no date; Minervini et al. 2003; Lee and Hur 2017; Mat Amin 2017; Fan et al. 2022).

During the last few years, there is a tendency for increasing the use of plant protein crops directly for human consumption and, as a result, legumes like lupine are being investigated more and more as

alternatives to soy and animal protein (Vogelsang-O'Dwyer et al. 2020). An ancient type of pulse crop, lupin, is a member of the genus *Lupinus* and the family *Fabaceae* (Johnson et al. 2017). However, it is a new genus to modern agriculture, and the seeds have great potential for high-protein food, animal feed, fodder, and improved soil fertility (Lambers, Clements and Nelson 2013). Due to the seeds' high dietary fibre content, gluten-free status, lack of significant amounts of starch, and thus very low glycemic index, lupine seeds have recently attracted a lot of interest as a food for human health (Sipsas, 2008). There are more than 400 species worldwide, but *Lupinus albus* (white lupin), *Lupinus luteus* (yellow lupin), and *Lupinus angustifolius* (Blue lupin) are the three most prevalent species of domestic lupin. The protein content of the plant may vary from 35 to 40 %, depending on the species (Lo, Kasapis and Farahnaky 2021). The significant potential of this source was highlighted by two recent review publications that summarized the potential role of bioactive peptides from lupin (Okagu et al. 2021; Garmidolova et al. 2022).

Enzymatic hydrolysis of food proteins is an effective way to generate potent bioactive peptides. Herein, we used three types of proteases (alcalase, pepsin and papain) to hydrolyze lupin protein isolate (LPI) and to evaluate the ACE inhibitory potential of lupin hydrolysates. There aren't many investigations, as to the antihypertensive activity of lupin proteins, so in this study, we aim to reveal the inhibitory potential of *Lupinus angustifolius* proteins to inhibit angiotensin-converting enzyme and to describe the influence of the enzyme used on the inhibitory activity.

Materials and Methods

Materials. Lupin organic flour (*Lupinus angustifolius* L.) was purchased from the commercial chain. The chemicals included: O-Phthaldialdehyde (OPA), di-Na-tetraborate decahydrate sodium tetraborate, sodium dodecyl sulphate (SDS), 2-mercaptoethanol, hydrochloric acid (HCl), 1,4-dithiothreitol (DTT), Bovine serum albumin (BSA), phosphate-buffered saline (PBS), angiotensin-converting enzyme (ACE), Sodium hydroxide (NaOH), Borate buffer, Potassium phosphate buffer, Hippuryl-histidyl-leucine (HHL), papain (A3824), alcalase (*Bacillus licheniformis*), pepsin (from porcine gastric mucosa, P7000, Sigma-Aldrich, St. Louis, MO, USA)

Sample preparation. The flour was defatted using 2-propanol in a 1:4 w/v ratio for 4 h at room temperature. Next, it was centrifuged at 6000 x g for 20 min, the residue was collected and the process was done twice.

The protein was isolated using alkaline extraction. The defatted lupin flour was suspended in distilled water 1:10 (w/v) and the pH was adjusted to 9 using 1M NaOH. The suspension was stirred for 1 h at room temperature before being centrifuged for 30 min at 4250 x g. On the residue, the extraction process was carried out twice. The protein samples were processed for further analysis by being lyophilized. In a subsequent study, we evaluated three biological activities of lupin hydrolysates using the same methods for protein extraction and additional hydrolysis (Garmidolova et al. 2022).

Enzymatic hydrolysis of lupin protein isolate. Five % (w/v) lupin protein extract was dissolved in appropriate buffers and prepared for enzymatic hydrolysis and pH was adjusted to each enzyme's optimal conditions (Table 1), following the product data-sheet. The suspension was continually agitated after the addition of the enzyme, and the temperature and pH were maintained constant.

Enzymatic hydrolysis of LPI is monitored for 0.5, 1 and 2 h for each of the following proteases: pepsin, papain, and alcalase. Three distinct enzyme/substrate ratios (E/S-0.5%, 1%, and 2%) were also investigated for each enzyme. After 20 minutes of heating at 95°C, the hydrolysed samples were cooled in order to end the enzymatic process. The samples were brought down to a pH of 4.5 before centrifugation at 4℃ for 15 minutes at 4250 x g. A negative control, protein lupin dispersion without enzyme, was performed under identical conditions. Afterwards, the lupin hydrolysates were lyophilized for further use.

Protein assay. The Lowry method was used modified to measure the protein content (Lowry et al. 1951).

Degree of hydrolysis. Each hydrolysate's DH was determined in triplicate using the method previously described by Nielsen (Nielsen et al. 2001) with serine as a reference. The following procedure was made to prepare the OPA solution:

Table 1. Enzymes employed in this study: sources and characteristics

Enzyme	E/S ratio, %	pH	T, ℃	Activity	Biological source
Alcalase	0.5 % 1% 2%	8	65	Serine endoprotease	*Bacillus licheniformis*
Papain	0.5 % 1% 2%	7	65	Cysteine endoprotease	Papaya (*Carica sp.)* latex
Pepsin	0.5 % 1% 2%	2	37	Aspartic endopeptidase	Porcine (*Sus domesticus*) gastric mucosa

Table 2. Abbreviations of hydrolysates and hydrolysis conditions for each enzyme

Sample	E/S, %	Duration of enzymatic hydrolysis, min
L0.5%30´	0.5	30
L0.5%60´	0.5	60
L0.5%120´	0.5	120
L1%30´	1	30
L1%60´	1	60
L1%120´	1	120
L2%30´	2	30
L2%60´	2	60
L2%120´	2	120

75 ml of distilled water was used to dissolve 3.81 g of di-Na-tetraborate decahydrate and 0.1 g of SDS. Then, 0.08 g of OPA was dissolved in 2 ml of 95% ethanol and quantitatively added to the solution above. 0.088 g of dithiothreitol (DTT) was added to the solution after washing it with distilled water. A standard serine solution was made (0.4758 meqv. L^{-1}). The lupin hydrolysates solutions were diluted with water to obtain samples with different concentrations. A spectrophotometer, SPECTROstar Nano Microplate Reader, was used to measure the absorbance at 340 nm after each sample (100 µl) was combined with 750 µl of OPA reagent and vortexed for 10 s (BMG LABTECH, Ortenberg, Germany). Using Nielsen' approach (Nielsen et al. 2001), the percentage of the DH is computed.

For calculating DH, we used the following formula:

$$\% DH = \left(\frac{h}{htot}\right) * 100 \quad (1)$$

Where, h_{tot}-indicates the overall amount of peptide bonds discovered in lupin hydrolyzates per protein equivalent, and h represents the amount of hydrolyzed peptide bonds, h = (serineNH2-β)/α meqv.g^{-1} protein). The composition of the amino acids used as raw materials affects the constant values β (slope of calibration through linear regression) and α (degree of dissociation of the α-amino group). According to theoretical general values for unexamined raw material, the h_{tot} factor was 1.0, 4.0, and 8.0, respectively (Nielsen et al. 2001). Afterwards, serine NH2 was calculated using the equation (*2*):

$$SerineNH2=(OD_{sample}-OD_{blank})/(OD_{standard}-OD_{blank})*0.9516meqv/L*V_{sample}*100/M_{sample}*PC_{sample} \quad (2)$$

Serine NH2 = meqv serine-NH_2/g protein; OD_{sample} - sample absorbance value; OD_{blank} - blank absorbance value; $OD_{standard}$ - standard absorbance value; V_{sample} - volume of sample solution (L); M_{sample} - weight of sample (g); PC_{sample} - protein content of sample (%)

ACE inhibitory assay. High-performance liquid chromatography (HPLC) was chosen to evaluate the

ACE-inhibitory activity of lupin hydrolysates. A method proposed in the literature by (De Leo et al. 2009) was used with slight modifications using Hypuryl-L-histidyl-L-leucine (HHL) as substrate. Briefly, 50 µl of 2.17 mM solution of hippuril-L-histidyl-leucine (HHL) dissolved in 50 mM borate buffer (pH 8.3) was mixed with 10 µl of peptide fraction (appropriate dilution) and 10 µl of 0.05 $U.ml^{-1}$ ACE dissolved in 50 mM potassium phosphate buffer, pH 8.3 at 37℃ for 30 min. The reaction was stopped by 100 µl of 1N HCl. The same procedure conditions were used to compare samples to a blank. The method was adapted with a C18 column (CORTECS C18, 2,7 µm, 2,1 × 150 mm). The mobile phase included 50% methanol and 0.1% trifluoroacetic (TCA) acid, the injection volume was 20 µl, the wavelength 228 nm and the flow rate was 0.8 $ml.min^{-1}$.

The basis for evaluating ACE inhibition is a comparison of HA (hippuric acid) in the presence or absence of an inhibitor. The percentage of ACE inhibition is calculated using the formula below given the peak area of HA:

$$\%\ Inhibition = \left[\frac{(Ak-As)}{Ak}\right] * 100 \qquad (3)$$

where, A_k – peak area of the sample without inhibitor and A_s – peak area of the sample with peptide fraction.

The amount of peptide required to inhibit 50% of an enzyme's activity, or IC_{50}, is the standard unit of comparison for biological activity. The value of the IC_{50}, after it has been determined, is frequently used to evaluate the biological activity of hydrolysates or peptides from one work to another. The nature and concentration of the reaction's substrate, the volume, the amount of the enzyme, the type of inhibitor employed to stop the process, and the methods used to detect the reaction's products are all important reaction characteristics that have a significant impact on the IC_{50}.

Determination of peptide concentration. The peptide concentration in the hydrolyzed samples was measured according to Lowry method (Hansen 1951).

Statistical analysis. The means of each experiment and analysis were performed in triplicate. Correlation and regression analysis were used to statistically analyze the experimental data. The coefficient of determination R^2 was used to test various regression models, including linear, logistic, exponential, polynomial, and power-law, and to identify the most accurate one. The ANOVA test and independent sample T-test using the software of SPSS were used to analyse the experimental data. P value < 0.05 was considered significant. Microsoft Excel 2019 was also used to execute some of the statistical calculations (Microsoft Corp., Redmond, WA, USA).

Results and Discussion

Degree of hydrolysis (DH) of lupin protein hydrolysates. The most used parameter for comparing various proteolytic processes is the DH, which provides information about the percentage of cleaved peptide bonds in a protein hydrolysate. During the enzymatic hydrolysis peptide bond between the amino and carboxyl groups of two adjacent amino acids is cleaved. This results in peptides of different sizes and free amino acids. The conditions of proteolysis and the type of enzyme have a significant influence on the DH (Mirzapour-kouhdasht et al. 2022). The authors also discuss the relationship between molecular size and the content of certain amino acids in peptides, which has an impact on biological activity.

Alcalase is an endopeptidase which breaks peptide bonds from C-terminal amino acids (Hunsakul et al. 2022). Pepsin, the stomach's major enzyme, is active in an acidic environment with a pH range of 1.3 to 2.0, as at pH 1.3 and its cleavage is more specific and preferentially breaks down proteins at position P1 or P1 at Phe, Tyr, Trp, and Leu (Kamran 2017). Because of the broad enzyme selectivity and specificity, the two enzymes can be applied with a variety of protein substrates resulting in a high degree of protein hydrolysis. In this paper, the results from alcalase and pepsin hydrolysis are presented in Fig. 1. As can be observed, alcalase hydrolysis displayed values from 9.89 ± 0.03% to 32.60 ± 0.02%, and pepsin protein cleavage varied from 6.85 ± 0.22% to 31.3 ± 0.49%. In our previous work, we investigated the DH of papain hydrolysates under the same conditions (Garmidolova et al. 2022). The results showed that DH varied from 9.06 ± 0.21% to 27.97 ± 0.37%.

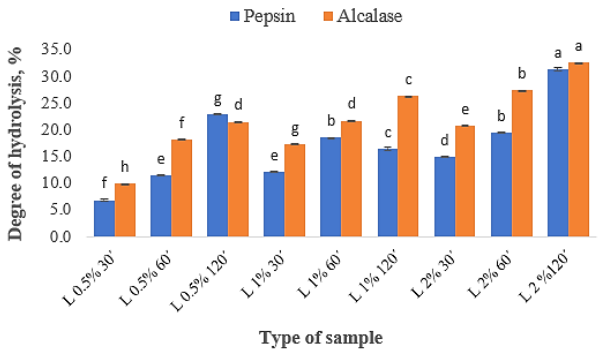


Figure 1. Degree of hydrolysis of lupin protein hydrolysates generated by pepsin and alcalase

a, b, c, d, e, f, g, h - indices showing significant differences ($p < 0.05$) between the mean values in the rows as analyzed by one-way ANOVA and the TUKEY test

Regarding Fig. 1 we observe the same tendency concerning DH for the two enzymes except sample L2%30. All samples showed higher DH with increasing hydrolysis duration and E/S ratio, indicating a significant release of peptide fragments during protein hydrolysis. Such behaviour may be caused by competition between the hydrolysis products and substrate, and denaturation of the enzyme, which decreases its activity (Sbroggio et al. 2016). At the beginning of hydrolysis catalytic sites, which are of prime importance for increasing the rate of hydrolysis, could be inaccessible. The enzyme gradually starts to attack the most sensitive peptide bonds and then hydrolyzes the remaining bonds until the hydrolysis is completed, depending on the amount of substrate available. In our case, alcalase cleaved more peptide bonds than pepsin. Similar results for alcalase (33.6%) are obtained by Sbroggio (Sbroggio et al. 2016) who investigated the influence of enzyme quantity and time of hydrolysis on the antioxidant activity of okara protein hydrolysates. Also, in the study of Jin et al. (2016), higher DH values were also obtained using alcalase as an enzyme to hydrolyze corn protein. In the work of Kamran (2017) who examined how pepsin hydrolysis influenced different biological activities of lupin hydrolysates, the results showed that in the first hour, DH was 31.11 ± 2.4% and at the end of the second hour 48.15 ± 3.3%, higher to our results (Jin et al. 2016).

The solo and combined effects of the E:S ratio and duration of hydrolysis on the degree of hydrolysis were analyzed by two-way ANOVA. E:S ratio and enzymatic hydrolysis duration's single and combined effects were calculated. Except for pepsin hydrolysis time, which did not affect DH, the data demonstrated that both the combined and individual effects of both parameters had an impact ($p < 0.05$) on the DH for both enzymes.

ACE-inhibitory activity of peptides from lupin protein isolates. We analysed protein hydrolysates of *Lupinus angustifolius* L. as a potential source of ACE inhibitory peptides. Inhibition of angiotensin-converting enzyme (ACE), an enzyme involved in blood pressure regulation, has a key role. There is evidence that low molecular weight peptides are able to inhibit the enzyme to a greater extent. The size of ACE-inhibitory peptides typically ranges from 2 to 30 amino acids (Paiva et al. 2017). However, scientific papers suggest that the presence of aromatic and chain-branched amino acids in peptide sequences can potentially enhance the inhibitory effect of dietary protein hydrolysates on ACE (Paiva et al. 2017). Numerous studies have demonstrated the efficacy of food ACE inhibitory peptides derived from food-protein, particularly legumes, in the prevention and treatment of hypertension (Daliri et al. 2018). In the scientific literature, there is a lack of knowledge on lupin-derived peptides on this topic. The results from our study are presented in Fig. 2.

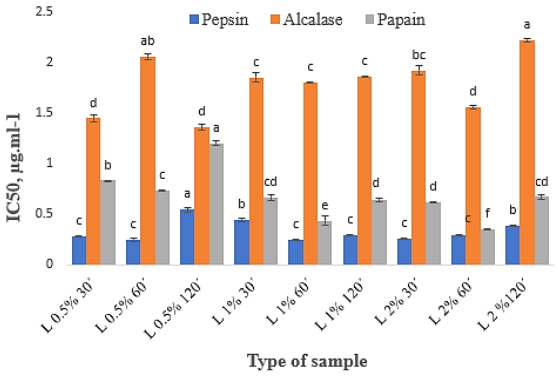


Figure 2. The ACE-inhibitory activity of lupin peptides

a, b, c, d, e, f, g - indices showing significant differences ($p < 0.05$) between the mean values in the rows as analyzed by one-way ANOVA and the TUKEY test.

According to the results, pepsin hydrolysates exhibited the highest inhibitory activity against ACE, followed by papain and alcalase hydrolysates.

The IC_{50} values for pepsin fractions varied from 0.26 ± 0.02 to 0.49 ± 0.01 μg.ml^{-1}, for papain in the range of 0.35 ± 0.11 and 1.15 ± 0.11 μg.ml^{-1} and alcalase resulted in values from 1.43 ± 0.03 and 2.23 ± 0.04 μg.ml^{-1}.

Regarding the pepsin hydrolysates, we can observe that statistically, there was no difference between samples L0.5%30, L0.5%60, L1%60, and L1%120; L2%30 and L2%60. In this case, we can suppose that the inhibitory activity is influenced to a greater extent by the type and length of the amino acid present and the degree of hydrolysis doesn't affect the inhibitory potential as much. Tyrosine, phenylalanine, tryptophan, proline, lysine, isoleucine, valine, leucine, and arginine have been found to significantly influence ACE binding in peptides, but further researches related to the identification of the varying peptides are necessary to prove the inhibition (Daskaya-Dikmen et al. 2017). The papain hydrolysates derived from lupin exhibited less enzyme inhibition potential. L1%60 and L2%60 inhibited it to the maximum extent (IC_{50} = 0.35 ± 0.01 and 0.43 ± 0.01 μg.ml^{-1}). The activity of proteins hydrolyzed with papain at an E:S ratio of 0.5% is reduced, and the IC_{50} values decrease as E:S concentration and hydrolysis time increase. At E:S = 0.5% ratio, the sample with the greatest inhibitory potential was L0.5%60, followed by L1%60 and L2%60 at E:S = 1% ratio and E:S = 2% ratio, respectively. When 60 minutes of enzymatic hydrolysis was performed at various E/S ratios, the hydrolysates exhibited the highest activity. The alcalase fractions exhibited the least inhibitory activity against ACE compared to the papain and pepsin fractions, which ranged from IC_{50} =1.43 ± 0.03 to 2.23 ± 0.04 μg.ml^{-1}. In contrast to the other two enzymes, alcalase hydrolysates demonstrated greater activity at E:S = 0.5%. Except for the L2%60 fraction, inhibition decreased at E:S = 1% and 2% ratios.

According to the statistical analysis performed, treatment of lupin proteins with pepsin, papain and alcalase resulted in peptide mixtures whose ACE-inhibitory activity was influenced by the E:S ratio, the hydrolysis time, the combination of both factors, and the degree of hydrolysis. The exception are pepsin fractions, which were not influenced by the last parameter, and alcalase hydrolysates in which the E/S = 1% ratio had no effect on the inhibitory capacity.

ACE-inhibitory peptides from natural sources are the subject of much research. Investigations from Boschin et al. (2014) evaluated also the ACE inhibitory activity of lupin hydrolysates and apart from the enzymes used, pepsin again exhibited the highest activity. Another study from Tawalbeh et al. (2023) evaluated the ACE inhibitory potential of pinto bean protein hydrolysate whose IC_{50} values varied from 0.22 to 0.26 mg.ml^{-1} and of purified ginko biloba hydrolysates (IC_{50} = 1.257 mg.ml^{-1}) (Ma et al. 2019). Regarding these results, we can consider that lupin-derived peptides exhibited higher inhibitory potential against ACE.

According to Hayes and Tiwari (2015), bioactive peptides originating from natural sources need larger amounts to be more effective than synthesized medicines. Nevertheless, our findings highlight the value of lupin as a nutritious food and suggest that lupin protein consumption may cause the digestive enzyme pepsin to produce ACE inhibitory peptides. However, it is important to note that further research is needed to fully investigate and evaluate their practical application.

Conclusion

The investigation's findings highlight the possibility of lupin proteins being a valuable source of peptides that block the ACE enzyme. In this study, we used three proteases to assess the inhibitory activity of the lupin protein hydrolysates, and we found that pepsin provided the lowest values of IC_{50} with a mean value of Δ0.33 ± 0.40 μg.ml^{-1}. Additional studies are needed to establish a direct correlation between *in vitro* and *in vivo* action due to the bioavailability of the ACE inhibitory peptides after oral administration. In conclusion, we may say that there is a wide range of food-derived bioactive peptides exerting antihypertensive activity and lupin is one of the plants with such an inhibitory potential which provide a wide field of action and exploring more of its characteristics will be helpful for the faster introduction in functional foods.

Acknowledgment

The authors want to thank prof. Atanas Pavlov, prof. Albert Krastanov and assoc. prof. Dasha Mihaylova for their scientific guidance and support. This publication has been carried out as part of the project: КП-06-М36/2, supported by the Bulgarian National Science Fund.

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