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Article

Isolation and characterization of antihypertensive peptides from soy bean protein

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Proteins and peptides are the most diverse biomolecules found in nature and make our interest due to their wide applications in food and pharmaceutical industry. Angiotensin Converting Enzyme (ACE) plays a major role in controlling blood pressure. The inhibition of ACE with peptides is a main target in the regulation of hypertension. The objective of the present study was to investigate the therapeutic potential of soy bean. This was accomplished by isolation of ACE inhibitory peptides using response surface methodology (RSM) and characterization of these bioactive peptides by mass spectrometry. 31 hydrolyzed fractions were isolated and evaluated for their ACE inhibition potential. Hydrolyzed fraction having highest ACE inhibitory activity was characterized by liquid chromatography-mass spectrometry (LC-MS) technique. RSM results showed maximum ACE inhibition potential (64%) by hydrolyzate was obtained at 45 ºC temperature, pH 8.0, E/S 0.2 in 2 hours hydrolysis time. Results of LC-MS analysis revealed Ser-Gly, Ser-Pro, Met-Ala, His-Ala, Lys-Pro, Phe-Thr, Met-Leu, Pro-Arg, Ala-Pro-Val, Pro-Ala-Leu, Val-Met-Gly, Pro-Leu-Val, Pro-Pro-Gln, His-Arg-Gly, Ser-Phe-Val-Leu, Ala-Val-His-Try, Arg-Thr-Val-Arg, His-His-Tyr-Leu-Val, Asp-Gly-Ala-Cys-Ser-Ala-Asn and Met-Val-Thr-Gly-Pro-Gly-Cys-His bioactive peptides in hydrolyzed fraction of soy bean. Our data provide evidence that response surface methodology is a good approach for isolation of antihypertensive bioactive peptides with more potent activity as nutraceuticals or pharmaceuticals. Therefore soy bean can be use for industrial production of pharmaceutical grade natural medicines for handling high blood pressure.

Keywords: Response surface methodology. Antihypertensive peptides. Optimization. Hydrolysate. ACE inhibition potential.

INTRODUCTION

Hypertension is a serious problem in developing countries and about 30% of adult population is suffering from it (Lopez-Exposito *et al*., 2012). It is closely associated with coronary heart disease (CHD). The ACE plays a central function in the rennin-angiotensin system (RAS). Renin-angiotensin system and the kinin-nitric oxide (NO) system physiologically control the hypertension. Based on the importance of ACE in the Renin-angiotensin system, ACE inhibitors have been used as antihypertensive agents (Ibrahim 2006). The worldwide statistics of hypertension shows that in 2000 the 972 million peoples were estimated with hypertension and this number is predicted to increase upto 60% of 1.56 billion in 2025 (Kearney *et al*., 2005). Hypertension is, therefore, a universal public health problem requiring significant attention with prevention and management. Although several potent synthetic drugs are utilized for the management of hypertension and other cardiac related diseases, but these are costly and possess various obnoxious side effects (Houston, 2013).

Antihypertensive bioactive peptides are suitable alternative due to their safe nature (Agyei, Danquah, 2012). The intake of plant peptide appeared with significant lowering of high blood pressure (Nirupama *et al*., 2015). These bioactive peptides have positive effects on body functions by inducing physiological responses such as antioxidant, antimicrobial, antihypertensive, cytomodulatory and immunomodulatory (Cheung

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et al., 2015; Blanco-Míguez *et al*., 2016). These responses of bioactives are the same as the targeted responses imparted by synthetic drugs. This diverse nature of peptides makes them suitable candidates for the development of curative agents (Lax, 2012). Bioactive peptides are gaining more attention by the scientific community, pharmaceutical corporations, consumers, physicians and patients because these are natural compounds possessing health providing properties (Borghi, Cicero, 2017). Pulses are protein-dense, high-fiber foods that contain variable amount of phytochemicals and antioxidants. Recent studies have considered soy bean as potential food due to its high protein contents and various phytochemicals. This study, therefore, was aimed to validate the effect of these bioactive peptides with the purpose of utilizing them as neutraceutical to efficiently control the increasing burden of hypertension with negligible side effects.

Enzyme hydrolysis is key step for isolation of antihypertensive peptides from soy bean proteins. Hydrolysis conditions like temperature, pH and enzyme to substrate ratio (E/S), and the hydrolysis time are important parameters for obtaining bioactive peptides. The hydrolysis is important to increase the production of peptides from laboratory scale to pilot scales having functional properties (Chiang *et al*., 1995).

MATERIAL AND METHODS

The protein of soy bean was extracted by using alkaline extraction method described by Tunc and Duman (2007). Dried Plant material was chopped, suspended in distilled water using a ratio of 1:15 (w/v) and maintaining pH up to 10.5 with 1M NaOH, stirring the mixture for 2 hours at 25 ºC to facilitate protein solubilization. The mixture was centrifuged at 3000 rpm for 15 min. The precipitates were mixed with distilled water (1:5 w/v) and the pH was adjusted again up to 10.5 and again centrifuged. This step was repeated three times. ACE inhibition potential of protein was assessed by the following equation:

$$ACEI\ (\%) = \frac{100\ [(A- B)-(C-D)]}{(A-B)}$$

Where, A represents absorbance at 228nm without inhibitor but with ACE. B stands for absorbance of reaction blank, C for absorbance of inhibitor with ACE, D for absorbance of reaction blank.

Isolation of bioactive peptides

Extracted protein was hydrolyzed by alcalase enzyme using central composite design (CCD). The levels used were coded as +1 (high), 0 (central point) and −1 (low). The factors and their levels have been shown in Table I. The extracted protein was dissolved in phosphate buffer (0.1 M, pH 7, 50 ºC), and alcalase enzyme was added using an E/S according to table 3.2. After adjustment to desired pH, the mixture was incubated at different temperatures. The enzymatic reactions were stopped by heating at 90 ºC for 15 min. At the end of the hydrolysis, all samples were centrifuged. The supernatant was collected and lyophilized (Pedroche *et al*., 2002). The hydrolyzates produced were tested for their ACE inhibition potential used for further characterization.

TABLE I – Reaction conditions for isolation of peptides

Factors	Level of factor		
	-1	**0**	**1**
Temperature (ºC)	35	45	55
Time (hour)	2	4	6
Enzyme/substrate	0.1	0.2	0.3
pH	7.0	8.0	9.0

Characterization of ACE Inhibitory Peptides

The bioactive antihypertensive peptides isolated from soy bean were identified by using liquid chromatography coupled with MS/MS through an electrospray ionization source. The hydrolyzed fraction was injected to the electrospray ionization (ESI) mass spectrometer with flow rate of 10ul/min. The range of 50 to 3000 m/z was selected in positive ionization mode for spectral analysis. The optimal values of the ESI-MS parameters for positive mode were: sheath gas and auxiliary gas 15 and 5 units per minutes respectively, spray voltage +4.0kV, capilliary voltage -20 V, capillary temperature 270 ºC and tube lense voltage of 100.51 V. The MS/MS fragmentation was done by using collision induced dissociation (CID) 25-30 units for each mass. The mass spectral data of molecular ions were processed by using Xcallibur software.

RESULTS AND DISCUSSION

The protein extracted from soy bean was hydrolyzed using following conditions of temperature (35, 45, 55 ºC), hydrolysis time (2, 4, 6 h), enzyme to substrate ratio (0.1, 0.2, 0.3) and pH (7.0, 8.0, 9.0). Peptides hydrolyzates were collected using these hydrolysis conditions. ACE inhibition potential of all hydrolyzates was evaluated to optimize the hydrolysis conditions for isolation of ACE inhibitory bioactive peptides. The percentage ACE inhibition potential of peptides isolated from hydrolysis of proteins from soy bean under different conditions has been presented in Table II. Temperature, pH, time and E/S ratio were selected as independent variables and ACE inhibition potential was response of bioactive peptides. Table II showed that hydrolysed fraction obtained at temperature 45 ºC, pH 8.0, E/S 0.2 in 2 hours hydrolysis time, showed maximum ACE inhibition potential (64%). Increasing hydrolysis time upto 4 hours decreased ACE inhibition potential (60%). Moreover, a further decreased ACE inhibition potential from 64% to 57% was noted by decreasing the pH from 8.0 to 7.0 without disturbing other reaction conditions.

TABLE II – ACE inhibition potential of hydrolyzates generated under different experimental conditions

i	35	2	0.1	7	48
ii	55	2	0.1	7	50
iii	35	6	0.1	7	55
iv	55	6	0.1	7	32
v	35	2	0.3	7	35
vi	55	2	0.3	7	42
vii	35	6	0.3	7	46
viii	55	6	0.3	7	52
ix	35	2	0.1	9	61
x	55	2	0.1	9	57
xxi	35	6	0.1	9	30
xii	55	6	0.1	9	35
xiii	35	2	0.3	9	45
xiv	55	2	0.3	9	53
xv	35	6	0.3	9	57
xvi	55	6	0.3	9	40
xvii	35	4	0.2	8	44
xviii	55	4	0.2	8	56
xix	45	2	0.2	8	64
xx	45	6	0.2	8	41
xxi	45	4	0.1	8	44
xxii	45	4	0.3	8	52
xxiii	45	4	0.2	7	57
xxiv	45	4	0.2	9	60
xxv	45	4	0.2	8	60
xxvi	45	4	0.2	8	60
xxvii	45	4	0.2	8	60
xxviii	45	4	0.2	8	59
xxix	45	4	0.2	8	60
xxx	45	4	0.2	8	60
xxxi	45	4	0.2	8	60

(continuing)

TABLE II – ACE inhibition potential of hydrolyzates generated under different experimental conditions

ACE inhibition potential of bioactive peptides isolated by enzyme hydrolysis of *Soy bean*protein isolate showed the effect of hydrolysis conditions (temperature (A), time (B), E/S ratio (C) and pH (D)) on response variable (% ACE inhibition) in terms of coded factors has been shown by regression equation (Equation 3).

$$\textbf{ACEI (\%)} = 127 + 3.56A + 35.72B + 320C - 2.0D - 0.0342A^2 - 1.106B^2 - 505C^2 + 0.58D^2 - 0.1000AB - 0.10AC + 0.012AD - 37.93BC - 2.000BD + 7.8CD \quad \textbf{(Eq 3)}$$

The regression equation revealed that the independent variables (A, B, C, D^2, AD, CD) with positive sign had positive effect and other independent variables (D, B^2, C^2, AB AC, BC, BD) with negative sign had negative effect on ACE inhibition potential of bioactive peptides isolated by enzyme hydrolysis of soy bean protein isolate.

Analysis of variance (ANOVA) was used to interpret the results and to evaluate the quality of fitted model selected for isolation of bioactive peptides. On the bases of *p* values (p=0.000) and *F* values (6.88) quadratic model was suggested to determine ACE inhibitory potential

of hydrolyzed proteins (Table III). These results were in accordance with the previous findings of Karki *et al.* (2011). The statistical analysis also showed that the lack of fit was not significant (p=0.097). The coefficient of determination R^2 (0.8576) and Adj. R^2 (0.7330) were in reasonable agreement with each other.

Analysis of variance (ANOVA) for response surface quadratic model has been given in Table 3. Results of ANOVA illustrated that time (B), interaction of time with E/S ratio (BC) and interaction of time with pH (BD) are the significant model terms. Present results are in agreement with the previous findings of Murado *et al*. (2010) and Kong *et al*. (2011) which showed the significant role of temperature, interaction of hydrolysis time with enzyme/substrate and pH in protein hydrolysis for isolation of peptides. It is revealed that temperature acts as limiting factor for ACE inhibition potential of hydrolyzates.

TABLE III – ACE inhibition potential of hydrolyzates generated under different experimental conditions

Source	DF	SS	MS	F-Value	P-Value
Model	14	2614.60	186.757	6.88	0.000
A-Temperature (ºC)	1	43.22	43.223	1.59	0.225
B- Time (h)	1	508.55	508.546	18.74	0.001
C- E/S (w/v)	1	37.56	37.556	1.38	0.257
D- pH	1	45.32	45.318	1.67	0.215
A2	1	30.43	30.425	1.12	0.305
B2	1	50.79	50.792	1.87	0.190
C2	1	37.52	37.519	1.38	0.257
D2	1	0.86	0.861	0.03	0.861
AB	1	64.00	64.000	2.36	0.144
AC	1	0.15	0.149	0.01	0.942
AD	1	0.25	0.250	0.01	0.925
BC	1	849.23	849.228	31.30	0.000
BD	1	256.00	256.000	9.44	0.007
CD	1	8.91	8.907	0.33	0.575
Error	16	434.11	27.132		
Lack-of-Fit	10	361.25	36.125	2.98	0.097
Pure Error	6	72.86	12.143		
Total	30	3048.71			
R2		0.8576			
R2 (adj)			0.7330		
R2 (pred)			0.4745		

The effect of temperature, time, pH and enzyme to substrate ratio (E/S) on enzymatic hydrolysis for isolation of bioactive peptides from protein extracted from soy bean was monitored by using response surface methodology. The three dimensional (3D) response surface plots were used to obtain optimal values of various factors on the ACE Inhibition response (Figure 1A-F). The interaction of temperature with time, pH and E/S has been shown in Figure 1A-C. It was observed from the Figure 1A, that ACE inhibition potential was increased by increasing temperature from 35 to 45 ºC. Further increasing temperature upto 55 ºC decreased the ACE inhibition potential of hydrolyzate. This can be explained by the loss of enzyme activity due to thermal inactivation of alcalase enzyme (Sindhu *et al*., 2014). Because at 45 ºC temperature, hydrophobic or proton donating amino acid residues inside the protein molecule get exposed and unfold which facilitate cleavage of peptide bonds, led to the increased ACE inhibition activity of bioactive peptides but further heating denatures the alcalase enzyme and prohibits its activity (Ren *et al*., 2008).

Figure 1B showed the effect of temperature and pH on ACE inhibition activity of soy bean hydrolyzate. ACE inhibition potential was increased by increasing pH from 7.0 to 8.2. An increasing ACE inhibition potential as a function of pH suggests that the compact primary structure of the protein may be partially unfolded at pH 8.2. pH value of 8.5 is suggested as optimum pH for the hydrolysis of proteins by alcalase (Costa *et al*., 2007). This may be assisting the enzyme to access the peptides in the primary sequence of the protein chains. Comparable result was observed in the previous finding by Pan and Guo, (2010).

The ACE inhibition value was increased by increasing the enzyme to substrate (E/S) ratio from 0.1 to 0.3, and maximum inhibition was noted at (E/S) ratio of 0.26 (Figure 1C). Figure 1D showed the combined effect of (E/S) ratio and hydrolysis time on ACE inhibition potential of hydrolyzate. It was observed from the 3D response surface plot that ACE inhibition potential was increased by increasing the E/S ratio as well as by increasing the hydrolysis time. Maximum ACE inhibition activity was obtained with E/S ratio of 0.20 in 2 hours hydrolysis time. Enzyme to substrate ratio is one of the most important factors and loading of protein substrate with high concentration of enzyme results in better hydrolyzate fractions. At 0.20 E/S ratio, the enzyme attacks the liable peptide bonds (Aziz *et al*., 2015) however, with the passage of time the substrate gets saturated with enzyme molecules and further increasing the enzyme concentration does not affect the hydrolysis. So due to the saturation of substrate molecules, further increasing the hydrolysis time have no effect on inhibitory activities of hydrolyzates.

The 3D plot of response surface suggested that interaction of pH and time was highly significant with ACE inhibition response of hydrolyzate (Figure 1E). Figure 1F showed the interaction between pH and E/S ratio. In the E/S range of 0.1- 0.3, the ACE inhibition potential increased with the E/S ratio upto 0.20. The effects of all reaction conditions on ACE inhibition potential of hydrolyzate showed that the optimal conditions of the enzymatic hydrolysis were 8.4pH, 45 ºC temperature, 0.20 E/S ratio and 2.0 hr hydrolysis time. These optimized conditions of pH, temperature, E/S and time were used for hydrolysis of protein extracted from soy bean and 63% ACE inhibition potential of hydrolyzate was observed. These results showed that proteins of soy bean are hydrolyzed into bioactive antihypertensive peptides.

Our results for optimization of hydrolysis conditions were consistent with previous findings of Xie *et al*. (2009) which showed the optimized conditions for soy bean hydrolyzates pH 7.83 and temperature 50 ºC, while high E/S ratio (4.5) and hydrolysis time (3.7 h) by protamex and trypsinase combination. Difference in optimized values of hydrolysis conditions in our experiment and previously reported may be due to difference in the peptide composition of the hydrolysate, difference in proteolytic mechanisms as well as difference in the structure of parent proteins due to the conditions used in processing (protein extraction) or due to the environmental condition in which the plant was grown (Vermeirssen *et al*., 2003). From this experiment it is clear that physiochemical parameters of the hydrolysis such as temperature, pH, time and E/S greatly influenced the release of ACE inhibitory peptides from soy bean proteins. That is why it is concluded that enzyme alcalase produced bioactive peptides having highest ACE inhibition potential *in vitro* and soy bean is a potential source of bioactive peptides.

The extracted protein of soy bean was hydrolyzed into bioactive peptides. These peptides were identified with LC-ESI-MS/MS in positive ionization mode presented in Table IV. These peptides include SG, SP, MA, HA, KP, FT, ML, PR, APV, PAL, VMG, PLV, PPQ, HRAG, SFVL, AVHW, RTVR, HHYLV, DGACSANG and MVTGPGCH.

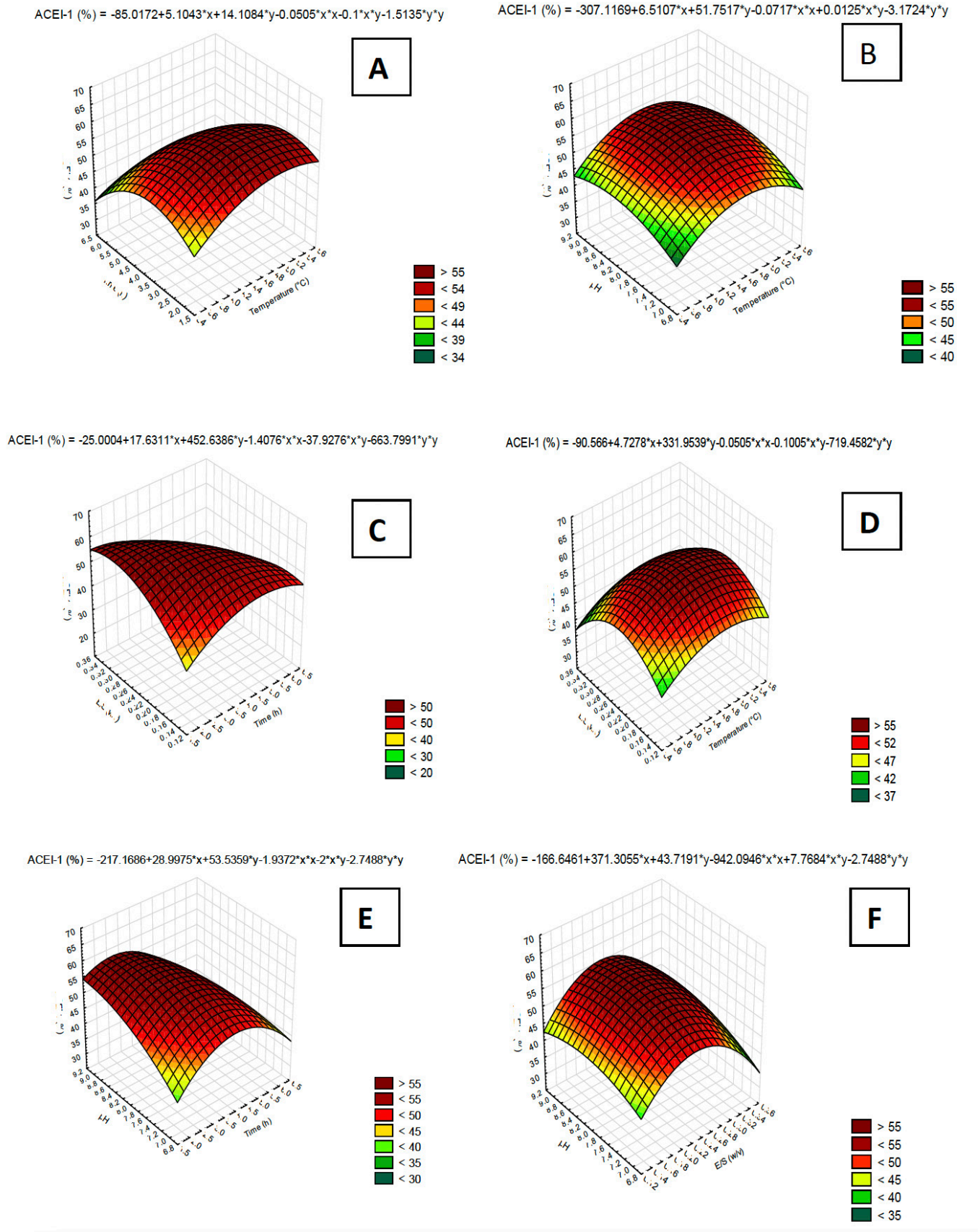


FIGURE 1 – ACE inhibition potential of soy bean hydrolyzates under different hydrolysis conditions. (A) Temperature and Time (B) Temperature and pH (C) Temperature and E/S (D) Time and E/S (E) Time and pH (F) pH and E/S.

TABLE IV – Name and structure of peptides identified in hydrolyzed fraction of soy bean in positive ionization mode

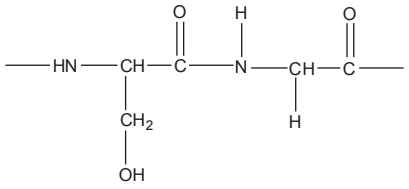
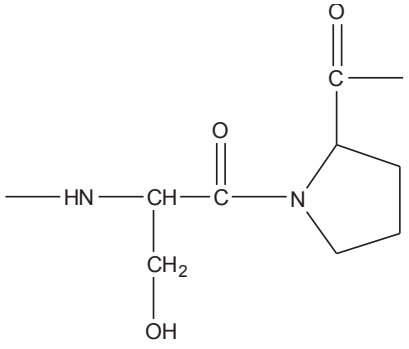
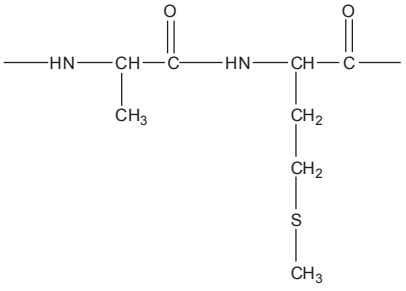
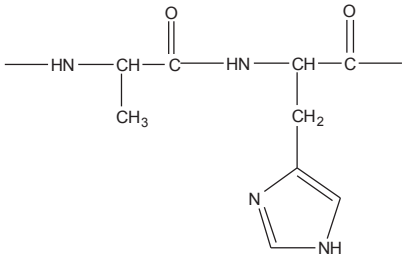
Sr. No.	Name of peptide	Structure of peptide
1	SG	
2	SP	
3	MA	
4	HA	

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TABLE IV – Name and structure of peptides identified in hydrolyzed fraction of soy bean in positive ionization mode

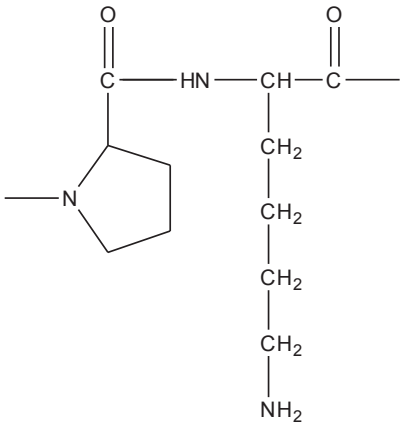
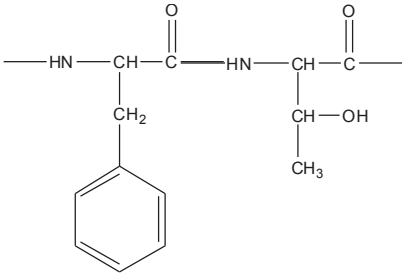
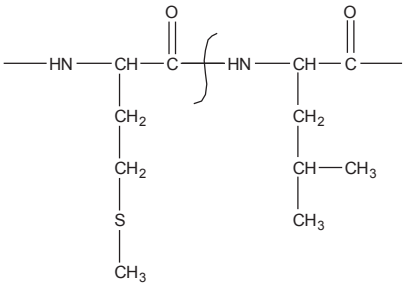
Sr. No.	Name of peptide	Structure of peptide
5	KP	
6	FT	
7	ML	

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TABLE IV – Name and structure of peptides identified in hydrolyzed fraction of soy bean in positive ionization mode

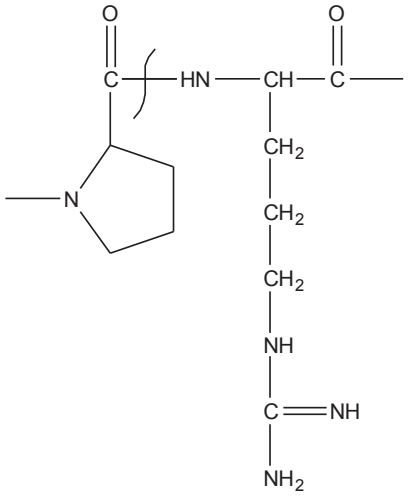
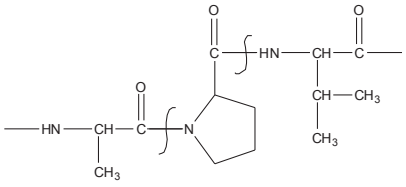
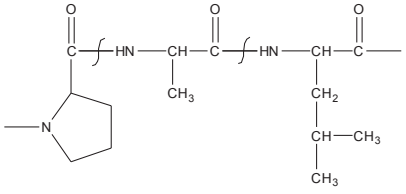
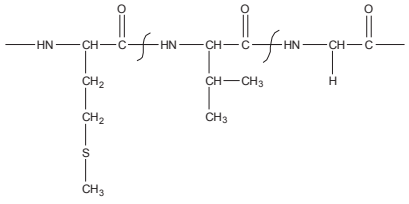
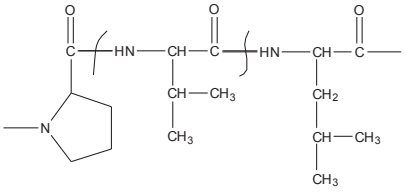
Sr. No.	Name of peptide	Structure of peptide
8	PR	
9	APV	
10	PAL	
11	VMG	
12	PLV	

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TABLE IV – Name and structure of peptides identified in hydrolyzed fraction of soy bean in positive ionization mode

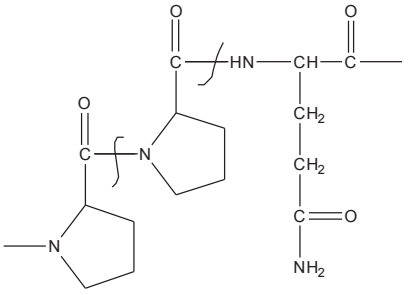
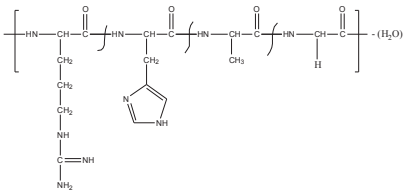
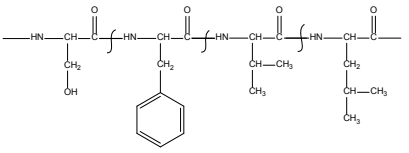
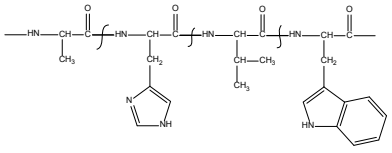
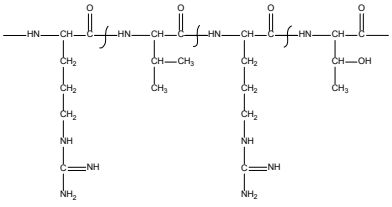
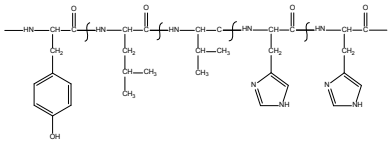
Sr. No.	Name of peptide	Structure of peptide
13	PPQ	
14	HRAG	
15	SLVG	
16	AVHW	
17	RTVR	
18	HHYLV	
19	DGACSANG	

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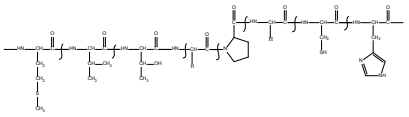
TABLE IV – Name and structure of peptides identified in hydrolyzed fraction of soy bean in positive ionization mode

Sr. No.	Name of peptide	Structure of peptide
20	MVTGPGCH	

LC-MS and LC-ESI-MS/MS analysis of the hydrolyzed fractions obtained by enzymatic hydrolysis of soy bean has been identified as dipeptides, tripeptides, tetrapeptides, hexapeptides, heptapeptides and octapeptides. The results of MS/MS analysis indicated that soy bean fraction contains biologically active peptides SG, SP, MA, HA, KP, FT, ML, PR, APV, PAL, VMG, PLV, PPQ, HRAG, SFVL, AVHW, RTVR, HHYLV, DGACSANG, MVTGPGCH. These bioactive peptides have also been identified from different sources and have been documented too. Met-Ala (MA) and His-Ala (HA) were identified in rapeseed hydrolysate (Schweizer *et al*., 2007), Pro-Leu-Val (PLV) was identified in A-casein (Balgir, Sharma, 2017) and Phe-Thr (FT) was identified in wheat bran (Tejedor *et al*., 2013). While Met-Leu, Pro-Ala-Val, Pro-Arg, Lys-Pro were identified from animal sources (Coutinho-Neto *et al*., 2013; Hong *et al*., 2008). In the present study, these all were identified first time in soy bean hydrolyzate.

Most of the bioactive peptides contain 2–20 amino acids residues exhibiting small molecular size. ACE inhibitory property relates with the peptides having hydrophobic amino acids (Lee *et al.,* 2014). Glycine, proline, leucine and phenylalanine are common amino acids having hydrophobic characters. In addition to the hydrophobic amino acids, the proline at the C-terminus also significantly contributes to ACE inhibiting activity. The present study revealed that proline (pro) is found in many of the ACE inhibitory peptides at the C-terminal including SP, KP, RP, AVP derived from soy bean hydrolyzate. Antihypertensive effect of bioactive peptides is due to presence of Valine, Histidine, Proline, Tryptophan or Methionine amino acids in the peptide sequence. Thus, the sequencing and structure of amino acid is related to ACE inhibition potential of antihypertensive bioactive peptides (Costa *et al*., 2007). This suggests that proline containing bioactive peptides may effectively interact with amino acid residues at the active site of ACE. This interaction leads to distort of Zn (II) and deactivates the ACE (Wu *et al*., 2015). This indicated that the bioactive peptides obtained by enzymatic hydrolysis of protein from soy bean have proline amino acid which make hydrogen bonding with Zn of ACE and inhibited its activity. Therefore, we can conclude that targeting the angiotensin converting enzyme with antihypertensive peptides, isolated from soy bean, provide a safe and economic treatment for hypertension.

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