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Isolation and characterization of novel antihypertensive bioactive peptides from *brassica napus* and angiotensin-converting enzyme (ace) inhibition potential

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Abstract: Present study aimed to explore the antihypertensive potential of bioactive peptides isolated from *Brassica napus* protein as inhibitor of angiotensin converting enzyme. Protein was extracted and assessed for antihypertensive potential. The extracted protein showed 72% antihypertensive activity/potential with IC_{50} value of 24±5.60µg/mL.Thirty-one fractions of peptides were isolated by hydrolyzing protein at different time intervals, pH, temperature and enzyme/substrate ratio. The antihypertensive potential of all isolated fractions was measured. It was found that only one peptide fraction exhibited significantly high (75%) antihypertensive potential. This hydrolyzed fraction was characterized through Liquid-Chromatography-Electrospray-Ionization-Mass-Spectrometry (LC-ESI-MS/MS). Eleven bioactive peptides were identified in hydrolyzate of *Brassica napus* which include Serine-Threonine, Methionine-Valine, Methionine-Leucine, Glutamine-Phenylalanine, Alanine-Threonine-Phenylalanine, Alanine-Leucine-Proline-Glycine, Valine-Alanine-Phenylalanine-Glycine, Aspartic acid-Proline-Methionine-Glutamine, Valine-Glutamine-Cysteine-Tyrosine, Methionine-Cysteine-Tyrosine-Tyrosine-Phenylalanine and Alanine-Leucine-Leucine-Alanine-Cysteine-Proline-Alanine. The current study showed that *Brassica napus* is an important food, having high amount of bioactive peptides with high antihypertensive potential, can control blood pressure very efficiently.

Keywords: Hypertension, antihypertensive peptides, enzyme hydrolysis, ACE inhibition potential, chromatography electrospray ionization mass spectrometry.

INTRODUCTION

Hypertension is documented as a major health issue due to its association with cardiovascular diseases (CVDs) (WHO, 2018). The report from the World Health Organization reveals that non-communicable and chronic CVDs are main cause of death worldwide (Mills *et al*., 2020). Physiologically, blood pressure is controlled by body renin-angiotensin-system (RAS). This system breaks angiotensinogen protein into angiotensin-I in the presence of renin. Ang-I is converted into Ang-II by the action of ACE. During hypertension, level of ACE increases which contracts blood vessel to produce high blood pressure. Therefore, ACE inhibition is important therapeutic target for making drugs to treat hypertension. From many years, synthetic drugs have been used to manage high blood pressure. However, these synthetic drugs show undesirable side effects at a high rate.

Due to increasing burden of hypertension, there is an urgent need for food with blood pressure lowering effect to treat hypertension Bioactive peptides from natural sources are valuable alternatives because of their cost effective nature and no side effects (Martinez-Villaluenga and Hernandez-Ledesma, 2020). Bioactive peptides were isolated and characterized from different plant and animal sources (Borghi and Cicero, 2017; Cicero and Colletti, 2017). These peptides are originated as new natural biological regulators, antioxidents and also used for the treatment of hypertension, diabetic and obesity due to their important structural characteristics, thus improving the quality of life (Huang *et al*., 2020).

Agriculture is the basis of Pakistan's economy and over the past 4 decades, *Brassica napus* have become one of the most important oilseed plant. This plant contains 50% protein with health-promoting properties. Several studies explored the effect of *Brassica* oil on platelet aggregation and inflammation of various diseases but the ACE inhibition potential of bioactive peptides from *Brassica napus* is still unexplored. Therefore, the present work was designed to evaluate the antihypertensive property of *Brassica napus*. The focus of the present research was to identify bioactive peptides to assess their structure-based relationship with ACE for making natural drugs against hypertension, which can prove to be a novel alternative to synthetic treatment options.

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MATERIALS AND METHODS

Analytical Instruments used for experiment

Spectrophotometer (Shimadzu Japan), pH meter (Hanna, Brazil), rotary evaporator (Japan), magnetic stirrer (DLAB, MS7-H550-Pro), centrifuge (SIGMA), oven (Memmert), electrospray mass spectrometer (LTQXL^{TM} linear ion trap Thermo Scientific, River Oaks Parkway, USA)

Chemicals used during experiment

Hippuryl-histidyl-leucine (Sigma-Aldrich), Hydrochloric acid (Merck), Ethyl acetate (Merck), PBS, disodium hydrogen phosphate (Riedel-deHaen), n-Hexane, Sodium borate (Fluka), Boric acid (Merck), Sodium hydroxide (Merck), Sodium chloride (DUKSAN)

Selection of plant

Brassica napus seeds were obtained from the local market of Faisalabad, Pakistan. The collected plant material was identified and ground into fine powder and used for study.

Extraction of Protein and Assessment of ACE Inhibition Potential

Protein was extracted using the alkaline extraction method explained by Bernardi *et al,* (2018). Powder of *Brassica napus* seeds was stirred with distilled H_2O (ratio 1:15). The pH of the mixture was maintained up to 10.5 by adding NaOH solution for 2 hours to solubilize the protein of the plant. Centrifuged the mixture at 5000 revolutions per minute (rpm), mixed the precipitates with distilled water, and repeated this step three times.

Evaluation of ACE Inhibition Potential

Extraction of ACE

ACE was extracted from rabbit lungs. Freshly slaughtered rabbit lungs were rinsed with normal saline (0.8%) and ground with phosphate buffer saline (PBS). Dehydrate the mixture using acetone under continuous stirring. The dried material was ground to a make powder and preserved in a polythene bag at 4°C (Chaudhary *et al.,* 2013). The lung acetone powder was dissolved in borate buffer (100mM, pH 8.3) and centrifuged for 45 minutes at 4000 rpm. Dialyzed the supernatant against borate buffer, lyophilized and preserved at -20^{o}C.

ACE Inhibition Assay

The ACE inhibition potential was measured according to a method reported by Belovic *et al,* (2013). 50µL of the enzyme (ACE) was incubated with the same volume of borate buffer at room temperature for 10 minutes. Freshly made substrate Hip-His-Leu in borate buffer (150µL, 8.3 mM) was mixed in above mixture and re-incubated further at room temperature. Reaction was stopped with 250µL of 1M HCl. Hippuric acid (HA) was separated from the mixture using ethyl acetate (1500µL) by centrifugation at 3000 rpm. The upper layer (750µL) was taken into the fresh test tube and air-dried. Solution of HA was made by dissolving HA in 1mL distilled water. The absorbance of this solution was determined at 228 nm with a UV-Vis spectrophotometer. The decrease in hippuric acid concentration in the sample concerning control was expressed as ACE inhibition potential (%). ACE inhibition potential was calculated by using the following equation deduced by Zheng *et al*, (2019):

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

Where A stands for absorbance of ACE. B for blank absorbance, C for protein/captopril in ACE and D for absorbance of a sample blank

Isolation of Bioactive Peptides

The bioactive peptides were obtained by alcalase hydrolysis of *Brassica napus* using different temperature conditions (35, 45 and 55°C), incubation time (2, 4, and 6h), E/S w/v (0.1, 0.2, 0.3) and pH (7.0, 8.0, 9.0). Protein was mixed with phosphate buffer (0.1 M, pH 7, 50^{o}C). Reaction parameters were maintained according to table 1 and incubated. The hydrolysis was stopped by heating the sample at 90^{o}C for 15 minutes and centrifuged at 6000 rpm for 15-20 minutes. The supernatant was obtained and lyophilized (Pedroche *et al.*, 2002). The hydrolyzates (peptide fractions) were assessed for the antihypertensive activity using above mentioned protocol.

LC-ESI-MS/MS Analysis of Peptides

The hydrolyzate/peptide fraction with high ACE inhibition potential was subjected to characterization with Liquid Chromatography Electrospray Ionization mass spectrometry technique. Peptide fraction was injected into the mass spectrometer using electrospray ionization source (LTQXL^{TM}, USA) with 10µL/min flow rate. The sample was analyzed in positive ionization mode using mass ranges from *m/z* 50-3000. The other parameters of ESI-MS were: +4.0 kV electrospray voltage; flow rate of the sheath and auxiliary gas was 15U/min and 5U/min respectively, 270^{o}C capillary temperature, -20.00 capillary voltage and 100.5 tube lens voltage.The fragmentation pattern was obtained by using collision induce dissociation 25-30U for each mass. Xcalibur software was applied to evaluate the mass spectral data of fragmented molecular ions (Thermo Fisher Scientific Inc, Waltham, USA). The peptides were identified at various *m/z* values. The chemical nature of peptide was proposed by MS and MS/MS fragmentation pattern (Nakano *et al*., 2009). MS/MS pattern of each peak in LC-MS/MS chromatogram was compared with standard spectra and literature studies.

STATISTICAL ANALYSIS

Data was analysed with SPSS software (version 21). All measurements were used in triplicates. The results were

obtained as average ±SE. IC_{50} values were calculated using the straight line equation.

RESULTS

Extraction of Protein and Isolation of Bioactive Peptides

Phytoprotien was extracted from the powder of *Brassica napus* seeds by stirring in distilled H_2O (ratio 1:15) and maintain the pH up to 10.5 by adding alkali. At the end the protein was isolated by centrifuging the mixture. Extraction process showed 4.12% yield of protein and 2.6% peptides.

Assessment of ACE Inhibition Potential

Protein was extracted from *Brassica napus* and evaluated as angiotensin-converting enzyme (ACE) inhibitor to find the actual bioactive peptides involved in ACE enzyme inhibition. The extracted protein showed 72% ACE inhibition potential.

Evaluation of ACE Inhibition Potential of Bioactive Peptides

The ACE inhibition potential (%) of hydrolyzate obtained from *Brassica napus* have been presented in table 1. Maximum ACE inhibition potential (75%) was observed by the fraction number xviii obtained at 55ºC, E/S ratio of 0.2 (w/v), and 8.0 pH in 4 hrs. The summary of ACE inhibition potential of bioactive peptides is shown in the table 1.

LC-ESI-MS/MS Analysis of Hydrolyzate of Brassica napus

Eleven peptides were identified in the positive ionization mode of Chromatography Electrospray Ionization mass spectrometry (LC-ESI-MS/MS) (fig. 1). The mass spectrum showed a peak at *m/z* 189 $[M+H]^+$. This peak was confirmed as Ser-Thr dipeptide (fig. 1) which produced fragmentation peaks at *m/z* 87.92 due to loss of $[M+H\text{-}Ser]^+$, at *m/z* 101.83 due to loss of $[M+H\text{-}Thr]^+$ from the molecular ion (table 2). Same MS/MS peaks were reported by Nakano *et al*, (2009). Peak appeared at *m/z* 231in MS spectrum was identified as Met-Val dipeptide $[M+H]^+$ (fig. 1) and confirmed through fragmentation ion peaks at *m/z* 132.00 which showed the removal of valine from parent molecule $[M+H\text{-}Val]^+$, at *m/z* 116.83 indicated the dehydration of methionine $[Met\text{-}H_2O]^+$, and at *m/z* 83.92 dehydration of valine $[Val\text{-}H_2O]^+$(table 2). These fragmentation peaks were similar to previously identified peaks (Nakano *et al*, 2009). The peak at *m/z* 246 was due to the Met-Leu dipeptide. MS^2 spectrum showed peaks at different *m/z* which confirmed dipeptide in hydrolyzed fraction. Dehydration of molecular ion $[M+H\text{-}H_2O]^+$was indicated by peak observed at *m/z* 229.08, leucine was confirmed by its elimination from parent ion $[M+H\text{-}Leu]^+$ indicated by peak obtained at *m/z* 132.92 and methionine from parent ion $[M+H\text{-}Met]^+$ at *m/z* 113.83 (table 2). The fragmentation pattern of these peaks was the same as reported earlier by Papayannopoulus (1995).

Dipeptide Glu-Phe was identified by a peak at *m/z* 275 in MS spectrum as parent molecule $[M+H]^+$ (fig. 1). Glutamic acid was confirmed by the appearance of a peak at *m/z* 146.00 by the loss of $[M+H\text{-}Glu]^+$ and at *m/z* 129.00 peak was appeared due to the removal of phenylalanine $[M+H\text{-}Phe]^+$, at *m/z* 111.92 by the elimination of $[Glu\text{-}H_2O]^+$ (table 2). Molecular ion peak $[M+H]^+$of tripeptide Ala-Thr-Phe appeared at *m/z* 317 which was confirmed by MS^2 peaks. Dehydration $[M+H\text{-}H_2O]^+$ was indicated by a peak at *m/z* 299.17, alanine and phenylalanine were removed from the parent molecule at *m/z* 246.17 $[M+H\text{-}Ala]^+$ and at *m/z* 171.00 [M+H-Phe] respectively (table 2).Tetra-peptide Ala-Leu-Pro-Gly (ALPG) was identified in hydrolyzate as indicated by molecular ion peak $[M+H]^+$ at *m/z* 340 in the mass spectrum (fig. 1) which were confirmed through further MS^2 peaks (table 2). The peak at *m/z* 322.17 was appeared due to the dehydration $[M+H\text{-}H_2O]^+$ from molecular ion, proline was removed $[M+H\text{-}Pro]^+$ at *m/z* 243.00, alanine and glycine were removed $[M+H\text{-}Ala\text{-}Gly]^+$ at *m/z* 211.08, leucine and glycine were eliminated $[M+H\text{-}Leu\text{-}Gly]^+$ at *m/z* 169.92, alanine and leucine were removed $[M+H\text{-}Ala\text{-}Leu]^+$ at *m/z* 155.92 and proline and leucine were removed $[M+H\text{-}Pro\text{-}Leu]^+$ at *m/z* 127.92 in MS/MS spectrum. Another tetra-peptide identified in hydrolyzate was Val-Ala-Phe-Gly (VAFG), which was indicated by parent ion peak $[M+H]^+$ at *m/z* 375.1 (fig. 1). Dehydration of this parent molecule $[M+H\text{-}H_2O]^+$was indicated by fragmentation ion peak at *m/z* 357, glycine, valine, and phenylalanine were removed from parent molecular ion at *m/z* 318.83 $[M+H\text{-}Gly]^+$, at *m/z* 276.08 $[M+H\text{-}Val]^+$, at *m/z* 228.08 $[M+H\text{-}Phe]^+$ respectively. While valine-alanine and phenylalanine-alanine were removed at *m/z* 205.00 $[M+H\text{-}Val\text{-}Ala]^+$ and at *m/z* 157.00 $[M+H\text{-}Phe\text{-}Ala]^+$ (table 2). Another tetra-peptideAsp-Pro-Met-Glu (DPME) was identified at *m/z* 472 $[M+H]^+$ (fig. 1). This tetra-peptide was confirmed by MS/MS peaks appeared due to removal of different amino acids from parent tetrapeptide i.e. $[M+H\text{-}Pro]^+$ at *m/z* 375.25, $[M+H\text{-}Asp]^+$ at *m/z* 357, $[M+H\text{-}Glu]^+$ at *m/z* 343.25 and $[M+H\text{-}Met\text{-}Glu]^+$ at *m/z* 211.08 (table 2).The fragmentation peaks appeared for DPME in this study agreed with the MS/MS peaks obtained by Choi *et al*, (2013) in another study.

Tetra-peptide Val-Glu-Cys-Tyr (VECY) was identified by parent ion peak $[M+H]^+$ at *m/z* 494 in the mass spectrum (fig. 1). VECY was confirmed by MS^2 peaks obtained by the removal of different amino acids from the parent molecule as indicated at *m/z* 395.33 $[M+H\text{-}Val]^+$ and at *m/z* 332.42 $[M+H\text{-}Tyr]^+$ (table 2). Hepta-peptide Ala-Leu-Leu-Ala-Cys-Pro-Ala (ALLACPA) appeared at *m/z* 640 $[M+H]^+$ (fig. 2).This heptapeptide was confirmed by MS/MS peaks that was appeared due to dehydration $[M+H\text{-}H_2O]^+$ at *m/z* 621.42 and *m/z* 568.42 due to loss of

Table 1: ACE inhibition potential of hydrolyzates of *Brassica napus* under various experimental conditions

Sr. No	Temperature (^{o}C)	Time (hr)	E/S (w/v)	pH	ACE Inhibition Potential (percentage)
i	35	2	0.1	7.0	25 ± 0.02
ii	55	2	0.1	7.0	34±0.022
iii	35	6	0.1	7.0	46 ± 0.03
iv	55	6	0.1	7.0	52 ± 0.08
v	35	2	0.3	7.0	57 ± 0.88
vi	55	2	0.3	7.0	60 ± 0.22
vii	35	6	0.3	7.0	64 ± 0.10
viii	55	6	0.3	7.0	67 ± 0.04
ix	35	2	0.1	9.0	12 ± 0.12
x	55	2	0.1	9.0	17 ± 0.02
xi	35	6	0.1	9.0	25 ± 0.02
xii	55	6	0.1	9.0	29 ± 0.16
xiii	35	2	0.3	9.0	46 ± 0.22
xiv	55	2	0.3	9.0	50 ± 0.56
xv	35	6	0.3	9.0	55 ± 0.12
xvi	55	6	0.3	9.0	67 ± 0.22
xvii	35	4	0.2	8.0	72 ± 0.06
xviii	55	4	0.2	8.0	75 ± 0.04
xix	45	2	0.2	8.0	15 ± 0.56
xx	45	6	0.2	8.0	29 ± 0.22
xxi	45	4	0.1	8.0	36 ± 0.12
xxii	45	4	0.3	8.0	48 ± 0.01
xxiii	45	4	0.2	7.0	59 ± 0.014
xxiv	45	4	0.2	9.0	67 ± 0.12
xxv	45	4	0.2	8.0	70 ± 0.02
xxvi	45	4	0.2	8.0	70 ± 0.04
xxvii	45	4	0.2	8.0	70 ± 0.14
xxviii	45	4	0.2	8.0	70 ± 0.16
xxix	45	4	0.2	8.0	70 ± 0.16
xxx	45	4	0.2	8.0	70 ± 0.14
xxxi	45	4	0.2	8.0	70 ± 0.14

Table 2: Peptides identified from *Brassica napus* protein through LC-ESI-MS/MS in positive ionization mode

S No.	Name of peptide	Type of peptide	MW	LC-ESI-MS/MS *m/z* $[M+H]^+$
1	Ser-Thr	Dipeptide	189	87.92, 101.83
2	Met-Val	Dipeptide	231	132.00, 116.83, 83.92
3	Met-Leu	Dipeptide	246	229.08, 187.00, 132.92, 113.83
4	Glu-Phe	Dipeptide	275	146.00, 129.00, 111.92
5	Ala-Thr-Phe	Tripeptide	317	299.17, 246.17, 171.00
6	Ala-Leu-Pro-Gly	Tetrapeptide	340	322.17, 243.00, 211.08, 169.92, 155.92, 127.92
7	Val-Ala-Phe-Gly	Tetrapeptide	375	357, 318.83, 276.08, 228.08, 205.00, 157.00
8	Asp-Pro-Met-Glu	Tetrapeptide	472	375.25, 357, 343.25, 211.08
9	Val-Glu-Cys-Tyr	Tetrapeptide	494	395.33, 332.42
10	Ala-Leu-Leu-Ala-Cys-Pro-Ala	Heptapeptide	640	621.42, 568.42, 470.42, 324.25, 296.17, 242.17
11	Met-Cys-Tyr-Tyr-Phe	Pentapeptide	706	602.58, 560.50, 470.42, 398.42

[M+H-Ala]. Two amino acids were removed from molecular ion at *m/z* 470.42 $[M+H\text{-}Pro\text{-}Ala]^+$ and four amino acids in different combinations were removed at *m/z* 324.25 $[M+H\text{-}Ala\text{-}Ala\text{-}Ala\text{-}Cys]^+$, at *m/z* 296.17 $[M+H\text{-}Ala\text{-}Cys\text{-}Pro\text{-}Ala]^+$, at *m/z* 242.17 $[M+H\text{-}Ala\text{-}Leu\text{-}Leu\text{-}Cys]^+$ (table 2). This fragmentation pattern has also resembled the pattern described by Seidler *et al.* (2010) in another study.

Pentapeptide Met-Cys-Tyr-Tyr-Phe (MCYYF) was indicated at *m/z* 706 $[M+H]^+$ (fig. 2). This was confirmed by MS^2 peaks, which were appeared at different m/z due to the removal of different amino acids from molecular ion i.e., at *m/z* 602.58 $[M+H\text{-}Cys]^+$, at *m/z* 560.50 $[M+H\text{-}Phe]^+$, two amino acids removed at *m/z* 470.42 $[M+H\text{-}Met\text{-}Cys]^+$ and at *m/z* 398.42 $[M+H\text{-}Tyr\text{-}Phe]^+$ in MS^2 spectrum (table 2).

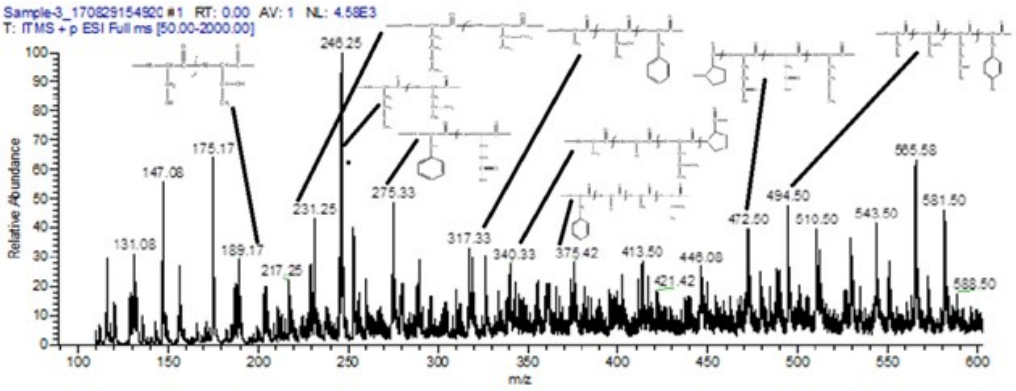

Fig. 1: LC-ESI-MS/MS spectrum (*m/z* 100-600) of the hydrolyzed fraction of *Brassica napus* generated through positive ionization mode

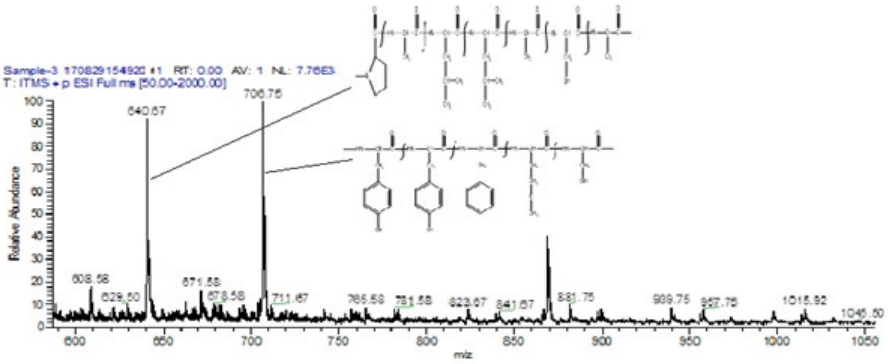

Fig. 2: LC-ESI-MS/MS spectrum (*m/z* 600-1050) of the hydrolyzed fraction of *Brassica napus* generated through positive ionization mode

DISCUSSION

Extraction of protein and peptides from various parts of plants is known very tedious job. Isolation of phyto-protein and peptides showed good yield which make the study pretty for further ACE inhibition potential evaluation. In the current study, the high ACE inhibition potential of *Brassica napus* protein may be due to the difference in plant species, nature and concentration of protein/bioactive compounds. The greater ACE inhibition potential of protein indicated that this bioactive compound was responsible for the inhibition of ACE which may be an exact phytochemical for the management of blood pressure. The antihypertensive potential of this plant was also previously reported due to the presence of other bioactive peptides (Capriotti *et al*., 2015). Protein extracted from *Brassica napus* showed IC_{50} value of 0.24 µg/mL, while plant protein/peptides generally have IC_{50} values of 15-312µg/mL (Daskaya-Dikmen *et al*., 2017) which proved that ACE inhibitory activity of *Brassica napus* protein is comparable with the peptides isolated from other plants. The very high ACE inhibition potential of this peptide fraction showed that no other chemical component like flavonoid, steroid, or alkaloid but only bioactive peptides of this plant may control hypertension by inhibiting angiotensin-converting enzyme. The effect of temperature was more pronounced as compared to other experimental conditions. It was 72% ACE inhibition potential was noted by changing the temperature from 55 to 35ºC; whoever, at 45ºC it was 70% by keeping all other parameters fix. Furthermore, the antihypertensive activity was decreased at high pH and low pH level. On increasing the pH from 8.0 to 9.0 ACE inhibition potential was reduced to 67% at 45ºC temperature and E/S ratio of 0.2 in 4 hrs. Similar ACE inhibitory activity (67%) of peptide fraction was obtained when the pH was further decreased to 7.0 but at a higher temperature (55ºC), E/S ratio (0.3), and in greater time (6 hrs). The results demonstrated the effect of operating experimental parameters on ACE inhibitory potential of hydrolyzate.

According to the structure-activity relationship (SAR) between peptide and ACE inhibition, the antihypertensive property of peptides depends upon amino acid sequence and their structure. Previously, bioactive peptides having hydrophobic amino acids exhibited ACE inhibitory properties (Hernandez-Ledesma *et al*., 2011). In this study, chromatographic data showed that hydrophobic amino acids were produced by the enzyme such as

alanine, histidine, valine, leucine, isoleucine, phenylalanine, proline, tryptophan, and methionine at the C-terminal site. Besides these amino acids, the proline at the C-terminal site also enhances the ACE inhibition potential of peptide (Shazly *et al*., 2017). The present study revealed the proline at the C-terminal site of many ACE inhibitory peptides. For normal ACE activity, Zn(II)is bound to histidine and glutamine amino acids present at active site of ACE which plays a vital role. Bioactive peptides form hydrogen bonds with zinc present at the ACE active site. This bonding led to the modification of Zn (II) structure, which ultimately diminishes the ACE activity. This mechanism suggests that bioactive peptides having proline effectively interacted with amino acid present at ACE active site. In the present study, the ACE inhibition potential due to protein contents/biopeptides is the novelty and the bioactive peptides of *Brassica napus* (ST, MV, ATF, DPME, ALLACPA, and MCYYF) were explored for the first time for their antihypertensive potential. Therefore, it is observed from this study that these novel antihypertensive peptides have important structural parameters required for SAR with ACE.

CONCLUSION

It was concluded that *Brassica napus* being a source of bioactive peptides can be used as a food component to act as ACE inhibitors in hypertensive patients. Since protein have physiologically positive role in the body although providing nitrogen and essential amino acids. Therefore, bioactive peptides identified in this study can be used to develop completely new products as a nutraceutical or functional food with the antihypertensive property.

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