

CHARACTERIZATION OF PEPTIDES FRACTIONS PRODUCED THROUGH ENZYMATIC HYDROLYSIS OF MEAT BYPRODUCTS FOR THEIR ANTIHYPERTENSIVE AND ANTIOXIDANT ACTIVITIES

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Meat industry produces huge amount of waste which have potential to be used as the valuable source of proteins and derived bioactive compounds. Dietary interventions have augmented the use of bioactive components with increased awareness for healthy life of individuals. Efforts have been endured to develop functional constituents that offer therapeutic responses towards physiological disorders. The current study was designed to investigate the therapeutic potential of meat byproducts hydrolysates derived after enzymatic hydrolysis with special reference to anti-hypertension and antioxidant activity. Meat byproducts were subjected to physicochemical assays and hydrolysis assessment were also carried out to estimate the release of peptides through RP-HPLC. Moreover, the hydrolysates containing bioactive peptides obtained after hydrolyses were further evaluated for antihypertensive and antioxidative activities. Liver and kidney showed higher enzymatic hydrolysis at maximum hydrolysis time. The ACE inhibitory activity of liver hydrolysate increased significantly with increase in hydrolysis time. The kidney hydrolysates have significantly higher ABTS radicals scavenging activity than liver and heart. The results indicated that hydrolysates of liver and kidney showed significantly higher oxygen radical absorbance capacity (ORAC) activity after given hydrolysis time. In conclusion, meat byproducts can be utilized for generation of functional bioactive peptides that therapeutic applications to cure human ailments.

Keywords: Meat byproducts, enzymatic hydrolysis, hydrolytic peptides, antioxidant activity, antihypertensive potential

INTRODUCTION

Meat is an animal flesh that offers high biological value protein, essential amino acids micronutrients and vitamins needed for healthy life (Williams *et al.*, 2006). Meat processing industry produces huge quantity of waste that poses potential pollution threats to the environment. Waste generated by meat industry can be used as valuable source for biological active compounds production to have value addition and utilization as nutraceuticals (Sohaib and Jameel, 2017). Protein waste have gained more attention among food processing sectors for production of bioactive peptides through value addition of animal by-products (Harnedy and FitzGerald, 2012). The possible route for the generation of bioactive peptides released during digestion of food and absorption done into the blood stream then they are transferred into different body parts from which they are utilized for the formation of new proteins and act as functional components (Segura-Campos *et al.*, 2011). Bioactive peptides in terms of structural confirmation comprise of few amino acids ranging from 2-30 units (Lafarga and Hayes, 2014) and have specific sequences that exhibit potential as functional and therapeutic food components (Erdmann *et al.*, 2008). Amino acid profiles of the organ meat (liver, kidney, heart, lung, spleen and bones)

exhibit diversity owing to the presence of connective tissue in large proportion accompanied by significant quantities of hydroxyl-proline, glycine, proline, traces of tyrosine and tryptophan (Bernardini *et al.*, 2011). Bioactivity of peptides depend on the sequence, composition and molecular mass of amino acids (Shahidi *et al.*, 2008). Peptides bioactivity is affected by the length of peptides and greater bioactivity has been reported for the small sized peptides (Moller *et al.*, 2008). Papain is a protease that shows extensive proteolytic activity for proteins including short chain peptides and amino acid esters especially for food and pharmaceutical applications (Uhlig, 1998). Bioactive peptide generation plays a vital role in the production of future functional foods as potential solution for processors to solve economic and environmental issues (Ren *et al.*, 2008). Chronic and infectious diseases can be effectively cured and managed by using bioactive peptides (Scanlon and Henrich, 2018) as natural and novel constituents through antihypertensive, antioxidant, antithrombotic and anti-inflammatory (Arihara and Ohata, 2010) anticancer activity. Proteins obtained from beef are known to be ultimate source of ACE-inhibitory peptides. According to literature a specific type of protein obtained from beef source i.e. sarcoplasmic protein extracts undergoes enzymatic hydrolysis are known to have ACE-inhibitory activity (Stadnik and Keska, 2015). Enzymatic

hydrolysis is one of the most common ways to extract bioactive peptides from whole protein source for showing antioxidative and cytotoxic effects against cancer cells (Udenigwe and Fogliano, 2017). Katayama *et al.* (2008) suggested that peptides for ACE inhibition were only generated from proteins like actin, myosin and sarcoplasmic. Some complex peptides were also obtained from muscle hydrolysates of bovine and porcine meat sources. They concluded that above mentioned proteins contain peptides affected blood pressure in different ways. Marine and meat-based peptides, possess some anti-cancer qualities by reducing cell proliferation and also possess some cytotoxic effect against invading cells of cancer (Mirdhayati *et al.*, 2016). Applications of bioactive peptides as an active component in nutraceutical and functional food industry is an innovative step in developmental trends and increasing thoughts of consumers to have healthy as well as nutritious food (Joana and Zbigniew *et al.*, 2013). The ultimate purpose of the study is to analyze the antihypertensive and antioxidant potential of peptides produced from enzymatic hydrolysis of meat by-products.

MATERIALS AND METHODS

The research was conducted at Meat Science and Technology Lab, National Institute of Food Science and Technology (NIFSAT), University of Agriculture, Faisalabad Pakistan and Department of Food Science, University of Massachusetts, Amherst, MA, USA.

Procurement of raw material: Meat byproducts (liver, kidney, heart) were purchased from the local butcher's shop for bioactive peptides generation and stored at freezing temperature (-40°C). The reagents and chemicals were procured from local suppliers of Sigma-Aldrich (Germany), Merck (Germany), and Oxide (UK).

Analysis of Meat byproducts: The samples of meat by-products were analyzed for pH by following the procedure of (Ong *et al.*, 2007) and acidity (method No. 974.05), protein (method No. 991.20) and ash content (method No. 945.46) by using their standard AOAC (2006).

Preparation of meat byproducts hydrolysates: Enzymatic hydrolysis was performed by following the method of Jang and Lee (2005) with minor modifications. Blended meat samples were homogenized and diluted with 1:10 w/v with phosphate buffer (0.02M) at pH 7.4 by using the homogenizer (VELP, Scientifica, OV5). Extracted protein was centrifuged at 11000 rpm for 30 min and collected supernatants were introduced with constant substrate to enzyme ratio 100:1. Hydrolysis of meat by-products was performed in triplicates at prescribed hour's interval (0, 2, 4, 6, 8 hours) in a shaking water bath (Mettler, D91126, Germany) at the agitation speed of 150 rpm. Inactivation of enzyme was done by heating at 90°C for 10 min and centrifugation was carried out at 11000 rpm for 15 min for

the separation of supernatant. Collected supernatants were freeze dried, vacuum packaged and stored at -40°C for further analysis to check their bioactive capacity.

Degree of hydrolysis of meat byproducts: Hydrolysis of meat byproducts was determined by following the method of Stadnik and Zbigniew (2013) and results were expressed as percentage of soluble nitrogen to total nitrogen.

Degree of Hydrolysis (%) = $\frac{100 \text{ TCA-soluble nitrogen}}{\text{Total protein}} \times 100$

Total protein

Quantification of bioactive peptides by using RP-HPLC:

Meat byproducts hydrolysates were further quantified through RP-HPLC system (Agilent Company, Series 1100, and USA). The RP-HPLC equipped with C18 column (25 cm x 4.6 mm, 5.0 µm, Ascetis) along with quaternary pump, with an auto sampler functionality and UV-visible detector (HP1050). Freeze dried meat byproducts proteins (20 mg) dissolved in 1 ml of solvent which contains tri-fluoroacetic acid (0.1%, TFA) that is further centrifuged at 11000 rpm for 15 min by using centrifuge machine (Eppendorf AG2233, Hamburg, Germany). The syringe filters of 0.45 micrometer were used for filtration to avoid any contaminations. Solvent A (water + 0.1% TFA) and Solvent B (TFA 0.1% into Acetonitrile) were prepared to attain linear gradient elution. Sample injection of 10 µL was done by auto sampler and peaks obtained at 215 nm of wavelength. The freeze-dried proteins were further analyzed for their given bioactive characteristics by following their respective protocols.

Activities of bioactive peptides: The generated bioactive peptides were further tested for their corresponding bioactivities according to their respective test methods.

Determination of angiotensin converting enzyme (ACE)

inhibitory activity: The inhibitory activity of generated bioactive peptides from meat byproducts were determined by the following the approved method of Cushman and Cheung *et al.* (1971). The hippuric acid forced by the action of the angiotensin-converting enzyme on HHL was extracted from the acidified solution into 1.5 ml ethyl acetate by vortex mixing for 15s. Final value for ACE inhibition was expressed as percentage by following the formula:

The angiotensin converting enzyme inhibitory activity was calculated by using the formula in percentage:

% Inhibitory Activity

$$= \frac{\text{absorbance of sample} - \text{abs of protein hydrolysates}}{\text{abs of control} - \text{optical density of blank}} \times 100$$

Determination of anti-oxidative capacity of meat

byproducts hydrolysates: The antioxidant activity of meat byproducts hydrolysates was measured by analyzing their scavenging activity. 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of meat byproduct hydrolysates was measured by following the protocol of Apostolidis *et al.* (2007), 2,2'-Azino-Bis-3-Ethylebenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity was determined by

following the method of Sah *et al.* (2014) while, Oxygen radicle absorbing capacity (ORAC) of protein hydrolysates were carried out by following the method of Davalos *et al.* (2004) with minor modifications.

Statistical analysis: All the experiments were run in triplicates to explore the bioactivities of generated bioactive peptides from meat by-products by using statistical techniques defined by Steel *et al.* (1997).

RESULTS AND DISCUSSION

Meat byproducts were analyzed for compositional analysis (pH acidity, crude protein, ash and crude fat content) and result are presented in Table 1. The results showing that composition of meat byproducts varied significantly. The pH and acidity of raw meat byproducts were found significantly different ranging from 5.86 to 6.21% while the acidity of meat by-products ranged from 0.41 to 0.46%. Seong *et al.* (2014) stated that variation in pH values among meat by products may be due to inherent characteristics and glycogen breakdown among by products. Ash contents of meat by-products varied from 0.89 to 1.20% while crude fat content of liver, kidney and heart ranges from 3.22% to 3.77%. The findings of fat percentage in meat by-products are in line to the study of Okumura *et al.* (2012). They concluded that fat content present in raw or un-cooked meat organs is lower than the fat content present in raw muscular portion of meat (Ba *et al.*, 2013). The highest protein content was found in liver meat followed by kidney and heart. The crude protein contents for meat byproducts were in range of 18.57 to 19.77%. The results of protein content in meat byproducts were in accordance to Ockerman and Basu *et al.* (2004) who reported that protein was found lower in cooked meat byproducts.

Table 1. Mean values for physicochemical composition of meat by-products (%).

Parameters	Physicochemical composition (%)		
	Liver (%)	Kidney (%)	Heart (%)
Protein	19.77±0.12	16.98±0.82	18.57±0.49
Fat	3.22±0.64	2.95±0.71	3.77±0.71
Ash	1.20±0.21	0.89±0.09	0.89±0.09
pH	6.21±0.10	6.50±0.17	5.86±0.05
Acidity	0.49±0.02	0.46±0.01	0.51±0.01

The cleavage of peptide bonds into its fractions without causing change in their amino acids sequence is hydrolysis and degree of hydrolysis is important parameter to check the maximum breakdown of bonds. Meat organs (liver, kidney and heart) and time of hydrolysis (0, 2, 4, 6, and 8 h) has highly significant effect on enzymatic activity of hydrolysate as given in (Fig. 1). The highest enzymatic activity was observed in liver varied from 7.31 to 30.55% which further increased significantly with increase in time of hydrolysis up

to 8 h while, minimum hydrolysis was observed in heart ranging from 3.51 to 20.23% which showed similar trend for increase in proteolytic activity with increase in hydrolysis time. Bernardini *et al.* (2011) assessed the effect of plant enzymes on degree of hydrolysis of bovine liver and observed that increase in time results in higher hydrolysis resulting in generation of small bioactive peptides that have anti-oxidative, anti-thrombotic and anti-hypertensive characteristics. Molecular weight of protein hydrolysates, amino acids sequence in resulting peptides, bioactivities are mainly affected by the substrate, proteolytic enzymes, and hydrolysis conditions provided for reaction (Balti *et al.*, 2011). Similarly, Tylor *et al.* (2002) investigated the effect of plant enzymes on sarcoplasmic and myofibrillar proteins and revealed that high degree of hydrolysis results in maximum solubility of proteins.

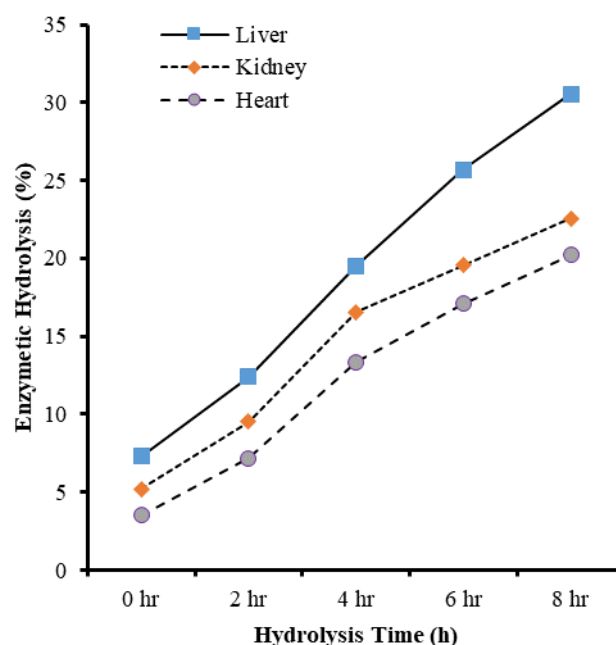


Figure 1. Interactive effect of meat by-products and time (h) on degree of hydrolysis (%).

The RP-HPLC profiling of meat byproducts hydrolysates obtained after different time of hydrolysis are presented in (Fig. 2). The effect of variables (meat byproduct hydrolysates and time of hydrolysis) showed highly significant ($p < 0.01$) effect on peak areas obtained after hydrolysis. It is expressed that hydrolysis times of different meat organs showed variation results in generation of peaks with different peak areas. Liver has highest peak areas at all hours of hydrolysis and the maximum peak areas (705.38 mAU*min) was obtained after 8 h of hydrolysis while minimum peak areas (76.23 mAU*min) was observed in sample without hydrolysis (0 h). Similar pattern of peak areas was noted for kidney and heart hydrolysates.

Hydrolysis in meat byproducts achieved is directly proportional to peak areas. Yoon *et al.* (2015) assessed the generation of bioactive peptides in salmon liver by hydrolyzing with alcalase enzyme. He quantified hydrolysates with RP-HPLC and observed that after 1 h of hydrolysis resultant range of peptides molecular weight varied from <50kDa and 100-500kDa. Differences in rate and pattern of hydrolysis could be due to diverse enzymes cutting sites as well as the availability of peptide bonds to each protease (Bordbar *et al.*, 2013). Bernardini *et al.* (2011) hydrolyze bovine liver sarcoplasmic proteins for 2 h and found that fractions with maximum peak areas showed higher bioactivities as maximum DPPH inhibition was observed with highest peak area. In general, bioactivities of peptides depend on the amino acids composition varying between hydrolysates, as well as may be affected by enzyme specificity and hydrolysis time (Chobert *et al.*, 1988).

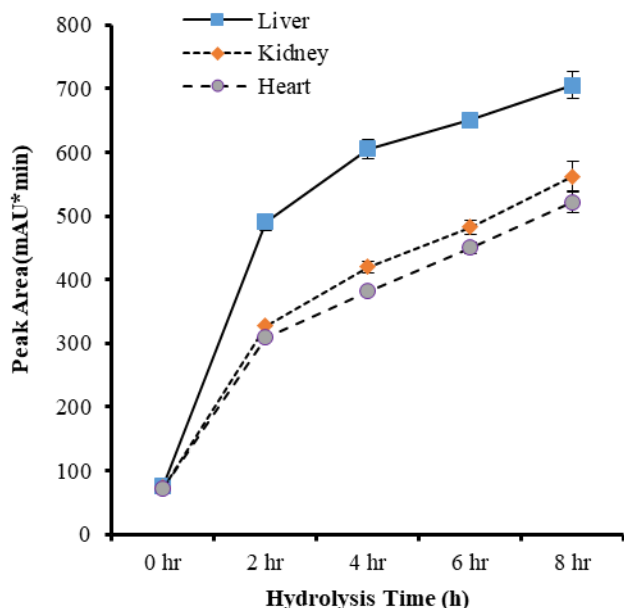


Figure 2. Interactive effect for meat by-products hydrolysates and hydrolysis times (h) on peak areas.

ACE inhibitors are used as the first-line treatment for the management of hypertension (Wang *et al.*, 2007). Angiotensin converting enzyme (ACE) increases the blood pressure by hydrolyzing rennin-induced decapeptide, angiotensin I to octapeptide angiotensin II (Jang and Lee, 2005). The results explicated in (Table 2) indicates that meat organs hydrolysate and time of hydrolysis have highly significant ($p < 0.01$) ACE inhibitory activity. The ACE inhibitory activity varied significantly from 7.36 to 34.01% for hydrolysates of meat organs at different hydrolysis time. Maximum ACE inhibitory value observed in liver hydrolysates while minimum values obtained in non-

hydrolyzed sample. These observations indicate that hydrolysis of different meat organs results variation in ACE inhibitory activity. Higher hydrolysis in meat byproducts achieved is directly proportional to more ACE inhibitory activity (Jang *et al.*, 2008). In a study performed by Jang and Lee (2004) they observed that beef liver sarcoplasmic proteins are hydrolyzed for generation of peptides that have sequence of valine at N-terminal are supposed to show high anti-hypertensive activity. Huang *et al.* (2015) also explored that meat byproduct hydrolyzed by papain enzyme leads to the generation of low molecular weight peptides that have anti-hypertensive properties. The fluctuation in the activity of ACE inhibitory peptides generated by the enzymatic hydrolysis may also be due to change in enzyme nature and pH of the hydrolysates as reported by Jang and Lee (2005). Low molecular weight peptides generated at early hours of hydrolysis exhibited high ACE inhibitory activity (Qian *et al.*, 2008).

Table 2. Mean values (%) for meat by-product hydrolysates (mg/mL) and hydrolysis time (h) on angiotensin converting enzyme inhibitory activity.

Hydrolysis time (h)	ACE inhibitory activity			SEM
	Liver	Kidney	Heart	
0	7.36 ^{cA}	5.34 ^{cB}	4.05 ^{dD}	0.12
2	21.45 ^{bA}	19.76 ^{bB}	15.36 ^{cC}	0.45
4	65.81 ^{aA}	59.24 ^{aB}	30.61 ^{bB}	1.62
6	68.32 ^{aA}	62.59 ^{aA}	32.96 ^{aA}	1.91
8	69.97 ^{aA}	63.58 ^{aA}	34.01 ^{aA}	2.11
SEM	1.98	1.50	0.63	

^{A-D}Row wise superscripts describe significant effect of meat by-products hydrolysates among treatments and ^{a-c}Column wise superscripts describe significant effect among hours.

Antioxidants present in food matrix scavenge the radicles by donating hydrogen ions to stabilize the free radicles (Baumann, 2008). The DPPH scavenging activity of meat byproducts hydrolyzed for different hydrolysis times are presented in (Table 3). The results explicated in Table 3 indicates that types of meat organs and hydrolysis time have significant effect on DPPH scavenging activity. The DPPH scavenging activity varied from 13.75 to 50.75% for hydrolysates of meat organs. The results elucidated that increase in hydrolysis time results in an increased scavenging of DPPH radicle. However, minimum DPPH scavenging activity was observed in sample without hydrolysis. Bernardini *et al.* (2011) extracted sarcoplasmic proteins from bovine liver muscle hydrolyzed for 2 h showed 95% of DPPH inhibition activity and observed that higher hydrolysis of proteins will result in more liberation of bioactive peptide. Je *et al.* (2009) also reported maximum antioxidant activity in tuna liver hydrolysates that show maximum 72% inhibitory activity. Different hours of hydrolysis containing low molecular weight bioactive

peptides showed maximum DPPH scavenging activity depending on nature of peptide and specificity of enzyme (Chang *et al.*, 2007). Increased antioxidant activity of hydrolysates depends on time of hydrolysis (Slizyte *et al.*, 2016), concentration of hydrolysates (Ngo *et al.*, 2011) and nature of enzyme (Haiping *et al.*, 2014).

Table 3. Mean values (%) of meat by-product hydrolysates (mg/mL) and hydrolysis time (h) on free radicle inhibition.

Hydrolysis time (h)	DPPH inhibition			SEM
	Liver	Kidney	Heart	
0	50.76 ^{cA}	20.72 ^{dB}	10.55 ^{dC}	0.65
2	71.36 ^{bA}	25.35 ^{cB}	15.17 ^{cC}	2.07
4	75.31 ^{abA}	27.87 ^{bB}	23.35 ^{bB}	1.68
6	80.45 ^{aabA}	31.81 ^{aB}	27.23 ^{aB}	1.57
8	70.72 ^{bA}	24.20 ^{cB}	13.75 ^{cC}	1.44
SEM	2.54	0.71	1.87	

^{A-C}Row wise superscripts describe significant effect among meat by-product hydrolysates and ^{a-d}Column wise superscripts describe significant effect among hours

The hydrogen donating ability of samples component states about ABTS radical scavenging antioxidant potential of samples mixture. It is perceived from the results given in (Table 4) that types of meat organs and hydrolysis time have significant effect on ABTS scavenging activity. Maximum ABTS scavenging activity was observed in kidney hydrolysate varying from 38.72 to 55.65% at different hours of hydrolysis while, heart hydrolysates ABTS scavenging activity varied from 35.52 to 44.57% for different hydrolysis times.

Table 4. Mean values (%) of meat by-product hydrolysates (mg/mL) and hydrolysis time (h) on ABTS radicle scavenging (%).

Hydrolysis time (h)	ABTS Radical Scavenging			SEM
	Liver	Kidney	Heart	
0	35.25 ^{cB}	38.72 ^{dA}	35.52 ^{bAB}	1.10
2	44.33 ^{bA}	45.65 ^{bcA}	41.27 ^{aA}	1.45
4	47.75 ^{abAB}	49.24 ^{bA}	43.22 ^{aB}	1.81
6	49.65 ^{aB}	55.65 ^{aA}	44.57 ^{aC}	1.65
8	38.42 ^{cB}	42.63 ^{cdA}	35.81 ^{bb}	1.03
SEM	1.44	1.30	1.57	

^{A-B}Row wise superscripts describe significant effect among meat by-product hydrolysates and ^{a-d}Column wise superscripts describe significant effect of meat by-product hydrolysates among hours

It is noted that hydrolysis of different meat organs at different hydrolysis times result in varied ABTS scavenging activity. Damgaard *et al.* (2014) studied the effect of bovine and porcine byproducts hydrolysates activity against ABTS radicles and found that bovine byproducts hydrolysates exhibited higher potency to scavenge the ABTS radicals.

Najafian and Babaji (2014) hydrolyzed *Patin* fish protein and observed that protein hydrolysates showed a significant ($p>0.05$) increase in inhibition of ABTS radicals. Similarly, increased inhibition of ABTS radicles was observed by Balakrishnan *et al.* (2011) for fermented shrimps and tanneries hydrolysate.

The mechanism of action of oxygen radicle absorbing capacity (ORAC) is based on the capacity of antioxidants present in food that scavenges the oxy-radicles which induces oxidation of 2,2-azobis (2-methylpropionamide) dihydrochloride (AAPH). Thermal decomposition of AAPH results in generation of peroxy radicals that further inhibits the fluorescence signals. Signal inhibition is quantified as area under curve (AUC) known as ORAC value (Salvin *et al.*, 2012). The results explicated that types of meat organ and hydrolysis time have significant oxygen radicle absorbing capacity as depicted in (Table 5). The ORAC values varied significantly from 385 to 387 μ M TEq for hydrolysates of meat organs. It is observed from the data that liver has significantly higher oxygen radicle absorbing capacity varying from 385 to 447 μ M TEq for different times of hydrolysis while oxygen radicle absorbing capacity of heart hydrolysates varied from 375 to 435 μ M TEq after different hours of hydrolysis.

Table 5. Mean values of meat by-product hydrolysates (mg/mL) and hydrolysis time (h) on oxygen radicle scavenging activity (μ mol trolox equivalent).

Hydrolysis time (h)	ORAC Activity			SEM
	Liver	Kidney	Heart	
0	385.00 ^{cA}	389.00 ^{bA}	375.00 ^{cA}	9.39
2	411.00 ^{bcA}	416.00 ^{abA}	408.00 ^{abcA}	12.20
4	422.00 ^{abA}	420.00 ^{abA}	411.00 ^{abA}	8.87
6	447.00 ^{abA}	443.00 ^{aA}	435.00 ^{aA}	7.10
8	398.00 ^{bcA}	397.00 ^{bA}	387.00 ^{bcB}	8.77
SEM	8.16	9.59	10.36	

^{A-B}Row wise superscripts describe significant effect among meat byproduct hydrolysates and ^{a-c}Column wise superscripts describe significant effect of meat byproduct hydrolysates among hours

Conclusion: In conclusions, meat byproducts can be used efficiently for generation of bioactive peptides. Meat byproduct hydrolysate showed highly significant ACE inhibitory activity after different hours of hydrolysis time. It showed that hydrolysates have bioactive compounds that are showing therapeutic potential to control high blood pressure. While, hydrolysis of meat byproducts also showed highly significant antioxidant activity. Liver hydrolysates have shown higher radicle scavenging and ORAC activities while kidney hydrolysate higher ABTS radicle scavenging activity. This study supports the use of meat byproducts for generation of bioactive compounds during hydrolysis that have potential to perform as an antioxidant that reduces the

reactive oxygen species and free radicles production furthermore control abnormal cell progressions and other complications.

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[Received 02 Jan 2019; Accepted 21 May- 2019
Published 8 Feb. 2020]