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Antioxidant and antimicrobial activity of protein hydrolysate extracted from porcine liver

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ABSTRACT

Protein hydrolysates were extracted from porcine liver using commercial proteases viz. alcalase, trypsin and papain. Porcine liver revealed appreciable amounts of protein (20.62%). Enzymatic hydrolysis of porcine liver hydrolysate (PLH) resulted in 23.56, 26.82 and 19.12% of degree of hydrolysis, respectively. Antioxidant activity such as 2–2 diphenyl–1-picrylhydrazyl (DPPH) and 2, 2-azino-bis-3-ethyl-benzthiazoline-6-sulphonic acid (ABTS) and ferric ion reducing power (FRAP) radicals were determined for PLH. All PLH samples showed slight decrease in the pH during hydrolysis while, they scavenged 42.27, 57.49 and 40.32% of DPPH radicals, respectively at sixth hour of hydrolysis. Trypsin hydrolysed PLH exhibited highest ABTS radical scavenging activity (86.79%) than alcalase hydrolysed PLH (74.62%) and papain hydrolysed PLH (70.63%). FRAP of PLH samples were found to be 13.69, 14.92 and 12.65% for alcalase, trypsin and papain extracted PLH samples, respectively. PLH obtained from trypsin hydrolysis showed highest antimicrobial activity followed by papain and alcalase PLH, respectively. However, all PLH have the potential to be a protein rich ingredient for use in formulated meat products and possible help in reduction of oxidative and microbial deterioration.

Key words: Antioxidant activity, Antimicrobial efficacy, Degree of hydrolysis, Porcine liver

Hydrolysate, a concoction of peptides and amino acids, is generated through hydrolysis by enzyme, acid or alkali treatment or fermentation. Protein hydrolysates are a low molecular weight peptides (2–20 amino acids) and good dietary source due to their excellent nutritional quality and therapeutic values (Bhaskar *et al.* 2007). These bioactive peptides generally scrambled and encrypted in the native or natural protein, and are usually quiescent within the structure of the protein. However, these bioactive peptides are obtained during gastro-intestinal/enzymatic or microbial digestion of parental protein. Bioactivity and functionality of the protein hydrolysates depend on the hydrolysis conditions and the source of the substrate as well as enzymes used. Non-purified protein hydrolysate can have certain benefits over those of purified peptides since the absorption of oligopeptides can be increased in the presence of sugar and amino acids. Whole hydrolysate exerts higher antioxidant activity than purified peptides (Sarmadi and Ismail 2010).

Peptides derived from food proteins have various functions such as antihypertensive, antioxidant and antithrombotic activities (Fukada *et al.* 2016). Meat by-products may be viewed as a potential starting substrate for the manufacture of high value-added products, including bioactive peptide containing hydrolysates (Bernardini *et al.* 2011). The extracted bioactive peptides exert antioxidant, antimicrobial and others functional activity (Jang *et al.* 2008, Liu *et al.* 2010). Enzymatic hydrolysis of the protein is an excellent method for the production of protein hydrolysates as the process can be easily controlled and is economical.

Therefore, researchers can use these high quality by-products obtained from the meat industry for the production protein hydrolysates which can be further used for development of healthier, nutraceutical and to enhance the storage stability of the perishable food items. Utilization of the meat industry by-products for the recovery of the protein hydrolysates from fifth quarter of the animal can solve the problem associated with the environmental pollution, proper disposal and economical loss.

Pork is the most popular meat form consumed worldwide. Pig population (FAO 2012) was 966.17 million. The total population of pig in India is 10.30 million as per 19th censes report and slaughter rate of pig is approximately 95% per annum. Percent yield of the liver from animals is approx. 1.2%; so the total amount of the production of the

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liver is approx. 117420 kg. Whereas the pork production is approx. 0.335 million tonnes per year and it contribute 0.035% of the total pig meat production. This abundantly available pork liver provides enormous scope and opportunities for its utilization.

For the first time, Marcuse (1960) studied that the peptides derived from dietary proteins have antioxidant properties. Since then, various sources of protein viz. whey and egg proteins (Sakanaka and Tachibana 2006), housefly larval proteins (Wang *et al.* 2013) and porcine hemoglobin (Alvarez *et al.* 2012) have been investigated to explore their antioxidant properties.

The present study was designed to utilize, explore and identify alterative cost effective protein hydrolysates that would open alternative avenues for meat by-products industry and give a boost to natural and healthier food additives enhancing consumer acceptability. Therefore, the objective of this study was to evaluate antioxidant and antimicrobial efficacy of porcine liver hydrolysate so that it can be used as a natural antioxidant and antimicrobial in comparison to synthetic antioxidant/antimicrobial ingredients used in meat industry.

MATERIALS AND METHODS

Enzyme alcalase (EC 3.4.21.62, activity ≥5 units/g protein) was procured from Sigma–Aldrich Chemical Co., Chandigarh, India and trypsin (EC 3.4.21.4, activity >250 USP units/mg protein) and papain (EC.3.4.22.2, activity ≥10 units/mg protein), were purchased from MP Bio-medicals, India. Fine chemicals such as 2, 2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma–Aldrich Chemical Co. India and 2, 4, 6-tripyridyl-s-triazine (TPTZ) was procured from MP Biomedicals, India. Other chemicals were of analytical grade from reputed companies and used without further purification. All solutions, prepared with double-distilled water, were kept at refrigeration temperature before use.

Enzymatic hydrolysis of porcine liver: Fresh porcine liver (Large White Yorkshire pigs of 9–12 months old, weighing 80–90 kg) was obtained from departmental slaughterhouse, packed in sterile low-density polyethylene (LDPE) and kept in deep-freeze (–18°C) till further use. Homogenized porcine liver was mixed (5:95 liver/water) in two different buffers to obtain different pH of the solution for optimum enzymatic activity (Table 1). Samples were heated in boiling water bath (Equitron, Model: 8414, Medica Instrument Mfg. Co., Mumbai, India) for 5 min to acquire pasteurization effect, as well as to denature the native enzymes and to unfold the proteins, which makes the protein more susceptible to enzymatic hydrolysis. For obtaining optimum hydrolysis, pH and temperature for the experiment were standardized by preliminary trials with the support of manufacturer's guideline as well as available literature. The enzyme substrate ratio (E: S ratio w/w) was kept constant (1:100) for all the enzymes. The hydrolysis was carried out by incubating the samples in stirred water bath and samples

Table 1. Optimum condition for enzymatic hydrolysis of porcine liver

Enzyme	Enzyme: Substrate ratio	pH	Buffer	Temperature (ºC)
Alcalase	1:100	8.0	Phosphate buffer	50
Trypsin	1:100	8.0	Phosphate buffer	37
Papain	1:100	6.5	Phosphate buffer	50

were collected at 0th, 2nd, 4th and 6th h of incubation. All hydrolyzed samples were immediately heated to 85ºC for 15 min in water bath to stop/terminate further enzymatic hydrolysis. Then, the hydrolysate samples were cooled and centrifuged in a refrigerated centrifuge (Eltek, Model: MP 400R, Elektrocraft (India) Pvt. Ltd., Mumbai, India) at 10,000 rpm for 25 min, supernatant was collected and stored at –20ºC until further use.

Proximate analysis: Moisture, protein, fat and ash content of the porcine liver was estimated by AOAC methods (AOAC 2000). To determine the moisture content, 10 g sample was dried at 100±5ºC to constant weight. Protein content (%) was estimated according to the Kjeldahl method. Factor 6.25 was used for conversion of nitrogen to crude protein. Fat percent was determined using Soxhlet apparatus. Total ash was estimated by using muffle furnace (550±5ºC for 6 h). An analysis of carbohydrate content in the samples was determined numerically (carbohydrate=100–(moisture+protein+fat+ash)).

pH measurement: The pH of porcine liver hydrolysate samples were recorded using combined glass electrode of Mettler Toledo pH meter (Model Five Easy$^{@TM}$ plus FEP 20, Switzeland). The pH of each sample was recorded just before heating to inactivate the residual concentration of enzyme.

Measurement of degree of hydrolysis: The degree of hydrolysis (DH) of porcine liver hydrolysate was measured by the percentage of solubilized protein in 10% (w/v) trichloroacetic acid (TCA), in relation to the total protein content of the sample according to Hoyle & Merritt (1994), with slight modifications. Aliquots (500 µl) of the hydrolyzed porcine liver protein were mixed with 500 µl of 20% (w/v) TCA solution to obtain the soluble and insoluble fractions in 10% TCA. After 30 min of rest, the mixture was centrifuged (Cooling Microfuge Model CM 12, Remi Elektro Technic Ltd, Vasai, India) at 3500 rpm for 15 min, and the soluble porcine liver protein content of the supernatant was determined by the method of Hartree (1972). The results were expressed as mg of protein. Bovine serum albumin was used as the standard. The DH was calculated according to the equation given below:

$$\text{DH (\%)} = \frac{\text{[Solubilized protein content in 10\% TCA (mg)}}{\text{Total protein content (mg)]}} \times 100$$

Determination of the antioxidant activity

2-2-azinobis-3ethylbenthiazoline-6-sulphonic acid ($ABTS^{+}$) radical scavenging activity: The

spectrophotometric analysis of $ABTS^+$ radical scavenging activity was determined as per method described by Salami *et al.* (2009). The method is based on the ability of antioxidants to quench the long-lived ABTS radical cation, a blue/green chromophore with characteristic absorption at 734 nm, in comparison to that of standard antioxidants. $ABTS^+$ was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^+$) was generated by reacting $ABTS^+$ stock solution with equal volume of 2.45 mM potassium persulphate ($K_2S_2O_8$) and keeping the mixture to stand in the dark at room temperature for 16 h before utilization. Prior to use, the stock solution was diluted with ethanol to an absorbance of 0.70 at t_0 (t=0 min) and equilibrated at 30°C exactly 6 min after initial mixing. About 1 ml of $ABTS^+$ working standard solution was mixed with 10 µl of hydrolysate/standard and absorbance was measured after 20 min (t_{20}) at 734 nm in multimode reader (Synergy H1 Hybrid Multi-Mode Micro-plate Reader, BioTek India, Mumbai). The $ABTS^+$ activity was calculated by using formula:

$$\text{ABTS activity (\% inhibition)} = [(0.7\text{-}At_{20})/0.7] \times 100$$

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity: The capacity to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical by added antioxidants in samples was estimated following the method of Brand-Williams *et al.* (1995) with slight modification. DPPH can make stable free radicals in aqueous or ethanol solution; however, fresh DPPH solution was prepared before every measurement. One millilitre of DPPH reagent (100 µM) was mixed with 0.25 ml of 0.1M Tris-HCl buffer (pH 7.4) and 25 µl of porcine liver protein hydrolysates sample in test tubes. The content was gently mixed and the absorbance at time t=0 min (t_0) was measured at 517 nm using multimode reader. The sample tubes were also incubated at room temperature under dark for measurement of absorbance at time t=20 min (t_{20}). Ethanol was used as blank. The free radical scavenging activity was calculated as decrease in absorbance from the equation:

$$\text{Scavenging activity (\% inhibition)} = 100\text{-}[(At_{20}/At_0) \times 100]$$

Ferric reducing-antioxidant power (FRAP) assay: The FRAP was assessed according to Benzie & Strain (1999) using multimode reader. The method is based on the reduction of the Fe^{3+}-TPTZ complex to the ferrous form at low pH. This reduction is monitored by measuring the absorption change at 593 nm. Briefly, 900 µl of freshly prepared working FRAP reagent (300 mM acetate buffer (pH 3.6), 20 mM ferric chloride solution and 10 mM TPTZ in 40 mM HCl in the ratio of 10:1:1) was mixed with 100 µl of hydrolysate sample and the absorbance at 593 nm was recorded using multimode reader after a 40 min incubation at 37°C. FRAP values were obtained by comparing the absorption change in the test mixture with those obtained from increasing concentrations of Fe^{3+} and expressed as mmol of Fe^{2+} equivalents per ml of sample. Ferrous sulphate was used as standard for standard curve preparation.

Antimicrobial activity (zone of inhibition)

Optimization of inoculation dose: The dose rate of the inoculum was standardized on the basis of bacterial number in the inoculums Pranoto (*et al.* 2005). The dose rate of the above mentioned microbial cultures was optimized in the range of 10^4–10^5 cfu/ml.

Well diffusion method: Protein hydrolysates extracted from the enzymatic hydrolysis of porcine liver by using different enzymes were tested. The pour plate method was used for determination of zone of inhibition for the selected food spoilage microorganisms. One millilitres of the test culture (10^4–10^5 cfu/ml) was uniformly distributed by pouring 15–20 ml of pre-sterilized media (anticlockwise and clockwise rotation) and allowed for solidification. In each plate, wells (10 mm diameter) were made using sterile cork borer. About 100 µl of each hydrolysate was poured into well onto solid media in nutrient agar for all the test organisms and incubated at 37ºC for 24 h. The diameters of inhibitory zone surrounding the wells were measured using digital vernier calipers.

Statistical analysis: Hydrolysis experiments of homogenized porcine liver protein were repeated three times and all the parameters were analysed in triplicate (n=9). Data were expressed as means with standard error. Two-way analysis of variance (ANOVA) was done by comparing the means by using Duncan's multiple range test (DMRT), at 95% confidence level using a SPSS package (SPSS 20.0 for Windows, SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Proximate composition of porcine liver: The mean values of proximate composition of porcine liver are depicted in Table 2. Porcine liver in present study had slightly lower moisture, fat and carbohydrate content but higher protein and ash contents than values reported for buffalo liver (Devatkal *et al.* 2004). However, Park *et al.* (1991) documented higher moisture value (75.9%) and lower fat per cent (3.26%) in sheep livers, respectively. These variations in proximate analysis might be due to the differences in species, breed, age, sex and variation in the level of nutrition. The porcine liver had a lower moisture and protein per cent and higher fat content than pork. This concorded with the observations of Park *et al.* (1991) that edible offals had significantly higher fat than muscle tissues of same species. The carbohydrate content of porcine liver (2.58%) was as per average values documented (USDA 1983) for hog liver. Edible offals including liver are considered as a rich source of the carbohydrates than muscle

Table 2. Proximate composition of porcine liver (n=9)

Parameter	%
Moisture	70.43±0.44
Protein	20.62±0.31
Fat	4.97±0.06
Ash	1.40±0.01
Carbohydrate	2.58±0.33

(Ockerman 1988). Hedrick *et al.* (1994) also documented that carbohydrate content in liver is approx. 2–8% on fresh basis and 0.5–1.3% in muscle on fresh basis. The ash content of porcine liver was comparable to bovine liver (Devatkal *et al.* 2004).

Effect of enzymatic hydrolysis on changes in pH: pH is an important parameter, which plays critical role in enzymatic hydrolysis of the protein. In the present study, the initial pH value for the optimum enzymatic hydrolysis was fixed at 8.0, 8.0 and 6.5 on the basis of literature survey and enzyme manufacturer guidelines for alcalase, trypsin and papain, respectively. The pH values in alclase and trypsin hydrolyzed sample decreased significantly ($P<0.05$) at 0, 2, 4 and 6 h hydrolysis (Table 3). However, in papain hydrolyzed liver sample, it decreased significantly ($P<0.05$) at 0 and 6th h, and remained similar during 2nd and 4th h. The variation in pH in papain hydrolysate porcine liver with alcalase and trypsin hydrolysate porcine liver might be due to the different buffer used for obtaining optimum hydrolysis. The drop in the pH values during entire enzymatic hydrolysis might be due to the release of more H^+ ion, when peptides bonds are cleaved (Ovissipour *et al.* 2013). Similar findings were also reported by Daroit *et al.* (2012) in ovine casein hydrolysates. During enzymatic hydrolysis due to the release of protons (H^+ ions) into the hydrolysate solution pH of the hydrolysate solution decreased.

Assessment of degree of hydrolysis: Enzymatic hydrolysis of porcine liver protein was carried out under the optimized pH and temperature condition for respective enzymes *viz.* alcalase, trypsin, and papain. The per cent degrees of hydrolysis (DH) of the porcine liver protein were 23.56, 26.82, and 19.12 for alcalase, trypsin, and papain, respectively at 6 h of incubation (Table 3). DH values were non-significant ($P>0.05$) among all groups at 0 h; however, in alcalase and papain treated liver samples, the DH values increased linearly with an increase in the hydrolysis time and differed significantly ($P<0.05$) at 2, 4 and 6 h, respectively. Whereas in trypsin treated porcine liver sample, the DH values increased significantly ($P<0.05$) up to 4 h; thereafter, the increase was not statistically significantly on 6 h. During initial period of hydrolysis, the rate of enzymatic reaction was very fast; however, with increase in hydrolysis time, the rate of reaction became slower. It might be due to decrease in the concentration of enzyme:substrate ratio and accumulation of the peptide in the vicinity of intermediate products and also due to competitive inhibition between un-hydrolysed protein and the peptides being constantly formed during hydrolysis. Hiidenhovi *et al.* (2005) also reported that the degree of hydrolysis of ovomucin hydrolysed with 10 different enzymes, including protamex, flavourzyme and alcalase were faster in initial hour of reaction. Chang *et al.* (2013) recommended the reaction time of 4 h due to higher activity up to this time.

Antioxidant activity of porcine liver hydrolysates: Antioxidant efficacy of porcine liver protein hydrolysates

Table 3. Comparison of pH, degree of hydrolysis and antioxidant activities of porcine liver protein hydrolysates with different enzyme and time interval (n=9)

Time (h)	Alcalase	Trypsin	Papain
	pH		
0	7.96 ± 0.01^{Db}	7.98 ± 0.01^{Db}	6.63 ± 0.01^{Ca}
2	7.89 ± 0.02^{Cb}	7.88 ± 0.01^{Cb}	6.55 ± 0.01^{Ba}
4	7.83 ± 0.02^{Bb}	7.83 ± 0.02^{Bb}	6.53 ± 0.0^{ABa}
6	7.77 ± 0.02^{Ab}	7.78 ± 0.01^{Ab}	6.50 ± 0.01^{Aa}
	Degree of hydrolysis (DH%)		
0	1.22 ± 0.03^{Aa}	1.24 ± 0.03^{Aa}	1.18 ± 0.20^{Aa}
2	13.21 ± 0.28^{Bb}	20.02 ± 0.42^{Bc}	11.74 ± 0.24^{Ba}
4	18.96 ± 0.46^{Cb}	26.20 ± 0.30^{Cc}	14.69 ± 0.18^{Ca}
6	23.56 ± 0.29^{Db}	26.82 ± 0.32^{Cc}	19.12 ± 0.46^{Da}
	DPPH (% inhibition)		
0	23.24 ± 0.25^{Ab}	24.38 ± 0.31^{Ab}	21.07 ± 0.60^{Aa}
2	33.64 ± 0.69^{Bb}	49.70 ± 0.47^{Bc}	30.54 ± 0.56^{Ba}
4	39.50 ± 0.64^{Cb}	55.26 ± 0.38^{Cc}	36.74 ± 0.71^{Ca}
6	42.27 ± 0.40^{Db}	57.49 ± 0.32^{Dc}	40.32 ± 0.25^{Da}
	ABTS (% inhibition)		
0	38.43 ± 0.47^{Aa}	38.43 ± 0.48^{Aa}	37.67 ± 0.34^{Aa}
2	55.95 ± 1.09^{Bb}	67.71 ± 0.76^{Bc}	48.24 ± 0.56^{Ba}
4	68.37 ± 0.27^{Cb}	85.26 ± 0.52^{Cc}	59.24 ± 1.56^{Ca}
6	74.62 ± 0.86^{Db}	86.79 ± 0.51^{Cc}	70.63 ± 0.57^{Da}
	FRAP (mM equivalent to $FeSO_4.7H_2O$)		
0	7.79 ± 0.22^{Aa}	8.33 ± 0.28^{Aa}	7.96 ± 0.10^{Aa}
2	10.29 ± 0.28^{Bb}	11.93 ± 0.19^{Bc}	9.51 ± 0.19^{Ba}
4	11.56 ± 0.38^{Ca}	13.97 ± 0.23^{Cb}	10.96 ± 0.33^{Ca}
6	13.69 ± 0.41^{Db}	14.92 ± 0.06^{Dc}	12.65 ± 0.39^{Da}

Values bearing same superscripts row-wise (small alphabets) and column-wise (capital alphabets) differ significantly ($P<0.05$). Values are expressed as mean±SE.

can be identified on the basis of two or more radical scavenging capacity assays due to the involvement of multi-dimensional antioxidant mechanisms. Therefore, in the present study, three radical scavenging activities viz. DPPH, ABTS and FRAP were used to assess *in-vitro* antioxidant potential of different porcine liver hydrolysates. Hydrogen-donating potency of antioxidant compounds were evaluated by DPPH radical scavenging activity, while single electron-transfer capabilities were determined by using ABTS. Fe^{+3} investigation in FRAP assay reflects the reductive antioxidant power of an antioxidant compound.

DPPH radical scavenging activity: DPPH radical scavenging activity assay is an extensively employed method for the quantitative evaluation of antioxidant efficacy of compounds as free radical scavengers or hydrogen donors. DPPH values increased linearly in all the porcine liver hydrolysate samples, irrespective of enzyme with the increase in the duration of hydrolysis (Table 3). However, during initial period of hydrolysis up to 2 h, increase in DPPH was exponential. It might be due to higher rate of hydrolysis during initial period. Hidalgo *et al.* (2012) also reported increase in DPPH scavenging activity with the increase of hydrolysis time. It might be due to relevant peptide structure, molecular weight and amino acid sequence to the radical-scavenging activity; the variation

in molecular weight, size, amount, composition and amino acids sequence of peptides, is attributed to varying enzyme/substrate/hydrolysis specificity. The results exhibited that alcalase and papain could terminate the radical chain reaction by converting free radicals into more stable products at 6th h; however, the trypsin terminate at 4 h hydrolysis. Mendis *et al.* (2005) and Rajapakse *et al.* (2005) also reported that the trypsin digested sample showed highest antioxidant activity against jumbo squid (*Dodicus gigas*) skin gelatine peptides and giant squid muscle peptides, respectively.

ABTS radical scavenging activity: $ABTS^+$ (2, 2 -azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical cation assay is an excellent tool for evaluating the antioxidant efficacy, and can be used to both lipophilic and hydrophilic compounds (Khantaphant and Benjakul 2008). As presented in Table 3, trypsin hydrolysates revealed highest $ABTS^+$ radical cation scavenging activities. The $ABTS^+$ radical scavenging activity increased significantly ($P<0.05$) with the increase in hydrolysis time up to 6 h for alcalase and papain. However, $ABTS^+$ activity increased significantly ($P<0.05$) up to 4th h in case of trypsin hydrolysate porcine liver sample, which might be either due to higher rate of hydrolysis during initial periods or enzyme/substrate ratio (thereafter limiting factor for the rate of reaction). Per cent inhibition of $ABTS^+$ radical cation ranged from 38.43–86.79% for trypsin hydrolysate, 38.43–74.62% for alcalase hydrolysate and 37.67–70.63% for papain hydrolysates, respectively for 0–6 h reaction time. Highest $ABTS^+$ activity was recorded for trypsin hydrolysate followed by alcalase and papain hydrolysate. The higher activity of trypsin hydrolysate is supported by higher DH, and the generated peptides might be varying in terms of amino acid composition, amino acid sequence etc. The activity of hydrolysates to scavenge $ABTS^+$ radicals depend on various factors such as the enzyme, DH, solubility of hydrolysates, class of peptides and existence of free amino acids (Phanturat *et al.* 2010). The finding of our study was in accordance with the report of You *et al.* (2010) for loach (*Misgurnus anguillicaudatus*) protein hydrolysates.

Ferric reducing antioxidant power (FRAP) activity: FRAP of enzymatic hydrolysate samples was expressed as mM equivalent to $FeSO_4.7H_2O$ (Table 3). Among the hydrolysate samples, the highest FRAP value was measured for trypsin hydrolysate (8.33–14.92) followed by alcalase (7.79–13.69) and papain (7.96–12.65). FRAP values varied significantly ($P<0.05$) with the varying enzymes and reaction time. In general, reductive antioxidant power (FRAP) of all the porcine liver enzymatic hydralysates was lower as compared to the $ABTS^+$ and DPPH scavenging capacities. Principle of the FRAP method is based on the reduction of the Fe^{+3}-TPTZ complex to the ferrous form at lower pH. The lower values of FRAP in alcalase and papain compared to the trypsin hydrolysate sample might be due to the lower capacity of liver hydrolysate to reduce ferric ion to its ferrous form. This result showed that the reducing power ability of liver protein hydrolysates mainly depend on the type of proteinase. Earlier some researcher also suggested that smaller size peptides released by enzyme digestion exhibited higher reducing power than higher molecular weight peptides (Ajibola *et al.* 2011). Khantaphanta *et al.* (2011) also reported the increase in FRAP activity of brown stripe red snapper muscle hydrolysate obtained using various proteases.

Antimicrobial activity of porcine liver protein hydrolysates (zone of inhibition): The antibacterial activity of porcine liver protein enzymatic hydrolysates was assessed against Gram-positive (*L. monocytogenes, B. cereus, S. aureus*) and Gram-negative (*E. coli*) bacteria (Table 4). Antimicrobial activity had positive correlation with period of enzymatic hydrolysis corresponding to DH, irrespective of type of enzyme. The extent of antibacterial activity of hydrolysate was assessed by zone of inhibition (mm). Trypsin hydrolysates showed highest antibacterial activity against *L. monocytogenes* (23.39±0.31 mm) followed by moderate inhibition of S. *aureus* (18.35±0.65 mm) and *E. coli* (18.17±0.36 mm) and least against *B. cereus* (16.79±0.35 mm) at 6 h of hydrolysis. Similarly, papain hydrolysates also exhibited stronger antibacterial efficacy against *L. monocytogenes* (17.05±0.32 mm), *E. coli* (16.88±0.48 mm), followed by *S. aureus* (15.13±0.29 mm) and *B. cereus* (13.39±0.32 mm). However, alcalase hydrolysates showed lowest antibacterial efficacy against *L. monocytogenes* (13.87±0.28 mm), *E. coli* (14.34±0.30 mm), followed by *S. aureus* (16.16±0.41mm) and B. cereus

Table 4. Evaluation of antimicrobial activity (zone of inhibition) of porcine liver protein hydrolysates by using different enzyme (n=9)

Time (h)	Alcalase	Trypsin	Papain
	Bacillus cereus (mm)		
0	ND	ND	ND
2	$9.74±0.35^{Aa}$	$11.15±0.18^{Ab}$	$10.40±0.27^{Aab}$
4	$11.49±0.31^{Ba}$	$13.75±0.48^{Bb}$	$11.69±0.35^{Ba}$
6	$12.62±0.29^{Ca}$	$16.79±0.35^{Cb}$	$13.39±0.32^{Ca}$
	Listeria monocytogenes (mm)		
0	ND	ND	ND
2	$11.13±0.22^{Aa}$	$13.32±0.24^{Ab}$	$10.44±0.37^{Aa}$
4	$12.46±0.32^{Ba}$	$22.42±0.37^{Bb}$	$13.58±0.44^{Ba}$
6	$13.87±0.28^{Ca}$	$23.39±0.31^{Cc}$	$17.05±0.32^{Cb}$
	Escherichia coli (mm)		
0	ND	ND	ND
2	$10.94±0.26^{Aa}$	$12.11±0.19^{Ab}$	$11.38±0.22^{Aab}$
4	$12.66±0.33^{Ba}$	$16.54±0.34^{Bc}$	$14.92±0.24^{Bb}$
6	$14.34±0.30^{Ca}$	$18.17±0.36^{Cc}$	$16.88±0.48^{Cb}$
	Staphylococcus aureus (mm)		
0	ND	ND	ND
2	$10.54±0.18^{Aa}$	$11.56±0.34^{Ab}$	$10.92±0.24^{Aab}$
4	$14.03±0.25^{Bb}$	$16.73±0.43^{Bc}$	$12.70±0.28^{Ba}$
6	$16.16±0.41^{Ca}$	$18.35±0.65^{Cb}$	$15.13±0.29^{Ca}$

Values bearing same superscripts row-wise (small alphabets) and column-wise (capital alphabets) differ significantly ($P<0.05$). ND, Not detected. Values are expressed as mean±SE.

(12.62±0.29 mm). The antibacterial activity for papain and alcalase porcine liver protein hydrolysates was comparable against *S. aureus and B. cereus* at 6th h; however, at 0 h hydrolysis, zone of inhibition was not detected for all tested bacteria. The antibacterial efficacy of porcine liver hydrolysate might be due to cationic property and hydrophobicity of peptides. The hydrophobic cationic peptides easily interact with anionic surface and internal cytoplasmic membrane of microbes, leading to change in membrane permeability of microbes and subsequently leaching out of the cellular content of microbes, leading to lysis of microbes. The lower MW peptides may have higher rate of interaction with cytoplasmic membrane than long chain peptides or higher MW peptides/protein. Antibacterial activity of the peptides/free amino acid might also be augmented due to its iron chelating properties. Wang (2003) also identified lower molecular weight antimicrobial protein (7.5 kDa protein and 6.0 kDa molecules) from porcine leucocyte, which inhibited the growth of *E. coli* and *S. aureus* in meat homogenate. Mine *et al.* (2004) postulated that digestion of hen egg white lysozyme with pepsin and trypsin, generated smaller molecular weight peptides (<5 kDa) with known antimicrobial activity. Similarly, Chakka *et al.* (2015) also recovered antimicrobial activity of chicken liver hydrolysate after the enzymatic and fermentation hydrolysis.

It can be concluded from the present study that antioxidant (ABTS, DPPH and FRAP assay) and antimicrobial activity of recovered porcine liver hydrolysates increased with degree of hydrolysis, irrespective of type of enzymes used and trypsin porcine liver hydrolysate showed considerably higher DH, antioxidant and antimicrobial activities. The degree and time of enzymatic hydrolysis varied with each enzyme and 6 h hydrolysis period was found highest for alcalase and papain, while optimum DH was accomplished within 4 h for trypsin hydrolysis. Thus protein rich meat by-products may be used as natural preservatives for extending the shelf-life of perishable meat products.

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