

In-vitro antioxidant and antibacterial properties of fermentatively and enzymatically prepared chicken liver protein hydrolysates

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Abstract Protein hydrolysates were prepared from chicken liver using fermentation and enzymatic hydrolysis. The lactic acid bacteria *Pediococcus acidilactici* NCIM5368 was employed in the fermentation process and a commercial protease (Alcalase® 2.5) was used in enzymatic hydrolysis. Chicken liver hydrolysates prepared by fermentation (FCLH) and enzymatic hydrolysis (ECLH) revealed appreciable amounts of protein [55.85 and 61.34 %; on dry weight basis, respectively]. Fermentation and enzymatic hydrolysis resulted in 14.3 and 26.12 % of degree of hydrolysis. Total antioxidant activity, reducing power, scavenging of superoxide, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis-3-ethyl-benzthiazoline-6-sulphonic acid (ABTS) radicals were determined for both FCLH & ECLH. FCLH & ECLH showed total antioxidant activity of 0.99 and 1.13 $\mu\text{g AAE mg}^{-1}$ proteins, respectively; while, they scavenged 96.14 and 92.76 % of DPPH radicals respectively. FCLH showed higher ABTS radical scavenging activity (32.16 %) than ECLH (19.29 %). Superoxide anion scavenging activity of FCLH & ECLH were found to be 95.02 & 88.94 %, respectively. Residues obtained after both treatments also exhibited antioxidant activities. FCLH reported highest antagonistic activity against *Listeria monocytogenes* (30 mm); while, ECLH showed antibacterial activity only against *Micrococcus luteus* (12 mm). Both hydrolysates have the potential to be a protein rich ingredient for use in formulated foods and possible help in reduction of oxidative stress.

Keywords Fermentation · Enzymatic hydrolysis · Chicken liver protein hydrolysates · Antioxidant · Poultry byproducts

Introduction

Poultry processing industry is one of the fast growing sectors in developing countries. In India, poultry industry is growing at an annual growth rate of 8–10 % and occupies 6th place in world chicken meat production with 2.21 million MT, whereas the total world production is 92.7 million MT (FAO 2012). Chicken meat processing industries produce edible (liver, gizzard, meat scraps including fat and blood) and inedible (intestine, feather, bones, feet and head) byproducts/wastes. Chicken liver is an edible byproduct and constitutes an excellent source of protein, minerals, vitamins and cholesterol (Kim 2011). Chicken liver constitutes nearly 4 % of body weight (Bowes and Julian 1988). It is estimated that 37 million MT of chicken liver is produced annually all over the world and creates disposal or pollution problems to organized poultry processing industries. Processing of the byproducts to convert them into a low value but utility product, or render them relevant to disposal costs, will add benefit to processors economically and reduce environmental pollution. Various technologies and processes are available to use these byproducts in animal feeds, but a growing market exists for preparation of protein hydrolysates for use as a functional ingredient or nutritional additive to low quality protein foods (Bhaskar et al. 2007a, 2007b).

Production of bioactive peptides by protein hydrolysis is one area that is gaining importance due to its wide range of applications in food, pharmacy and cosmetics (Radha et al. 2007). Hydrolysis of byproducts can be accomplished by biological and chemical methods. Fermentation among all biological methods and enzymatic hydrolysis over chemical

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methods are strongly recommended for production of protein hydrolysates (Rustad 2003; Jung et al. 2005). Bioconversion methods like fermentation involving lactic acid bacteria (LAB) for preservation of byproducts/wastes along with recovery of functional biomolecules have been of interest to develop eco-friendly methods of waste management. Fermentation is reported to be effective in recovering biofunctional molecules that have antioxidant and antibacterial properties (Sachindra and Bhaskar 2008) and has become an important tool in waste treatment.

Alternatively, enzymatic hydrolysis is also been considered as efficient for production of protein hydrolysates. Many researchers found that hydrolysis of proteins by enzymes facilitates better digestion with high yield and forms easily soluble forms of peptides. Enzymatic hydrolysis, tenders better control over hydrolysis process than autolytic hydrolysis and hastens the process than autolytic hydrolysis and gives better products with consistent quality (Liaset et al. 2000). Enzymatic hydrolysis is influenced by various factors such as pH, time, enzyme to substrate level and temperature. Especially, the enzyme employed in hydrolysis influences the degree of hydrolysis which affects the functional properties of the hydrolysates produced (Mullaly et al. 1995). Alcalase (an alkaline bacterial protease that is commercially produced from *Bacillus licheniformis*) has been suggested, for preparation of protein hydrolysates due to its advantages against pH and cost effectiveness, by several researchers (Bhaskar et al. 2007a, 2007b; Klompong et al. 2007).

Bioactive peptides generated from both fermented and enzymatically hydrolysed meat proteins exhibit various biofunctional properties such as antioxidative, anti-hypertensive and immunomodulatory properties depending on their sequence and amino acid composition (Pihlanto and Korhonen 2003). Many bioactive peptides derived from meat proteins are reported to have anti-hypertensive properties (Abdul et al. 2010). Reactive oxygen species such as hydroxyl, superoxide and peroxyl radicals are formed in human tissue cells and can result in extensive oxidative damage that leading to age related degenerative conditions, cancer and wide range of other human diseases. To solve this problem both synthetic and natural antioxidants have been widely used but synthetic antioxidants have been suspected to be responsible for toxicity when used on a long term basis (Ito et al. 1986). This has led researchers to consider potential natural biomolecules that prevent toxicity effects. Protein hydrolysates react rapidly with these free radicals and retard or decrease the extent of oxidative deterioration. Selective peptides of meat protein hydrolysates proved antibacterial against several pathogens

like *Listeria monocytogenes* Scott A, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Micrococcus luteus* and *Yersinia enterocolitica* (Adje et al. 2011). Many researchers have prepared protein hydrolysates with fish waste (Liaset and Espe 200, Bhaskar et al. 2007b), fish muscles (Klompong et al. 2007), chicken intestine (Jamdar and Harikumar 2005), chicken skin (Onuh et al. 2014) and sheep visceral mass (Bhaskar et al. 2007a); but, there are no reports available on preparation of protein hydrolysates from chicken liver by fermentation or enzymatic hydrolysis by a commercial protease (Alcalase®). In this context, the present study aimed at the preparation of chicken liver protein hydrolysates and evaluating their potential for antioxidant and antibacterial activity.

Materials and methods

Materials

Raw chicken liver (RL) was procured from the local meat market, transported to the laboratory under ice condition and stored at $-20\text{ }^{\circ}\text{C}$ until use. 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2-deoxy-D-ribose, α -tocopherol, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), pyrogallol red and Alcalase® 2.5 (a commercial protease obtained from *Bacillus licheniformis*) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Ethylenediaminetetraacetic acid (EDTA), Folin-Ciocalteu's phenol reagent, Hydrogen peroxide, ascorbic acid and α -tocopherol were purchased from Merck (Mumbai, India). D-glucose and NaCl (Salt) used for fermentation experiments were procured from Loba chemicals (Mumbai, India). Thiobarbituric acid (TBA) and all microbiological media (all were dehydrated media) were purchased from Hi-Media (Mumbai, India). *Pediococcus acidilactici* NCIM5368 isolated from fish waste (Jini et al. 2011) was used for fermentation experiments. The pathogenic bacterial strains (viz., *Bacillus cereus* F4433, *Escherichia coli* MTCC118, *Staphylococcus aureus* MTCC1430, *Listeria monocytogenes* MTCC157, *Yersinia enterocolitica* MTCC859 and *Micrococcus luteus* MTCC2452) used for antibacterial activity in this study were from the institute's culture collection (Food Microbiology Department, CSIR-CFTRI, Mysore). These strains were stored at $-80\text{ }^{\circ}\text{C}$ in brain heart infusion (BHI) broth with 20 % (v/v) glycerol. Cultures were propagated twice in fresh BHI medium before they were used. All other solvents and chemicals were of analytical grade unless otherwise mentioned.

Methods

General

Proximate composition of raw, cooked, fermented, enzymatically hydrolysed mass, hydrolysates and residue was estimated as per AOAC methods (AOAC 2000). Briefly, Moisture content was determined by oven-drying at 105 °C to constant mass. Protein content (dry weight basis) in all samples was analyzed by Kjeldahl method using Gerhardt Vapodest 30S (Made in Germany). Ash content was determined by combustion of the sample at 550 °C for 8 h. Fat content was determined by the method of Bligh and Dyer method (1959) which involved extraction with chloroform: methanol (2:1). The pH measurements were accomplished by directly immersing the combined glass calomel electrode into the sample using pH meter (Cyberscan 1000, Eutech, Singapore). Total titrable acidity (TTA) of fermented samples was estimated as per the method described in Bhaskar et al. (2007b) by determining the volume (ml) of 0.1 N NaOH required for increasing the pH of one gram of fermented mass to 8.0.

Preparation of chicken liver for fermentation and enzymatic hydrolysis

Cleaned chicken liver was minced in a mincer and steam cooked (~80 °C) for 10 min in order to inactivate endogenous enzymes and to kill spoilage microorganisms. Minced and cooked chicken liver was used as a substrate for fermentation and enzymatic hydrolysis.

Production of fermented chicken liver protein hydrolysates

Preparation of inoculum

The lactic acid bacteria culture *Pediococcus acidilactici* NCIM5368 was grown in 100 ml of MRS broth for 24 h at 37±2 °C in a shaking incubator (Labtech, Daihan labtech co. ltd, India) agitating at 120 rpm. The cells were collected in the form of pellet after centrifuging (Rotina 420R Cooling centrifuge, Denmark) MRS broth at 3000 g for 10 min. These cells were washed twice with sterile saline and resuspended in 100 ml of saline.

Fermentation

Known weight of cooked chicken liver was mixed with distilled water (1:1, w/v) and fermented under optimized fermentation conditions [sugar- 15 % (w/w), inoculum- 10 % (w/v) and NaCl - 2 % (w/w)] for 24 h at 37±2 °C in a shaking incubator with agitation (120 rpm) to attain

a desirable pH 4.2±0.2 (Metna 2013). The fermented mass was then centrifuged (2000 g for 10 min) to obtain the fermentation liquor as the supernatant that was collected and lyophilized to obtain Fermented chicken liver hydrolysates (FCLH). The residue after removing the fermented liquor was also lyophilized to obtain Fermented chicken liver residue (FCLR). Fermentation of chicken liver as followed in the current work is presented schematically in Fig. 1.

Enzyme hydrolysis

Known weight of cooked chicken liver was mixed with distilled water (1:1, w/v) followed by the addition of a commercial protease (Alcalase® 2.5) at 1.5 % (v/w of solids) level followed by thorough mixing of all the contents and then incubated under shaking condition (120 rpm) for 150 min at 45±2 °C in a water bath shaker (Rivotek, Selec TC344 India). After incubation, enzyme activity was terminated by heating the content mix at 90 °C for 10 min and allowed to attain room

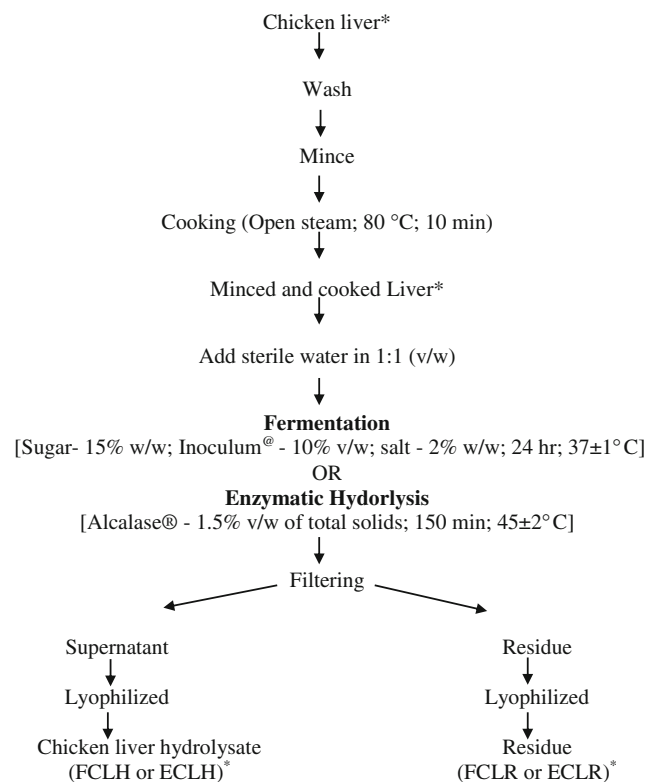


Fig. 1 Schematic representation of chicken liver hydrolysate preparation by fermentation and enzymatic hydrolysis. * Samples analysed for various parameters; @ : *Pediococcus acidilactici* NCIM5368, FCLH : Chicken liver hydrolysate prepared using Fermentation, ECLH : Chicken liver hydrolysate prepared by enzymatic hydrolysis, FCLR : Residue obtained after separation of fermentation liquor, ECLR : Residue obtained after separation of enzymatic hydrolysate

temperature. The hydrolysed mass was then centrifuged (2000 g for 10 min) to obtain the supernatant which was lyophilized to obtain enzymatic chicken liver hydrolysates (ECLH). The residue was also lyophilized to obtain enzymatic chicken liver residue (ECLR). Enzymatic hydrolysis of chicken liver as followed in the current work is presented schematically in Fig. 1.

Degree of protein hydrolysis (DH)

Degree of protein hydrolysis (DH) in the liquor resulting from fermented and enzymatic hydrolysed chicken liver was estimated as per the method published by Nana TH & John HM, (1994) and was calculated as follows

$$\text{DH\%} = 10\% \text{ TCA soluble Nitrogen in the sample} / \text{Total Nitrogen in the sample} \times 100$$

Antioxidant properties

Sample preparation for antioxidant assays

FCLH, FCLR, ECLH and ECLR samples in the form of powder were dissolved (10 mg/ml) in distilled water except in DPPH assay where methanol was used to dissolve the samples. This was used as sample for evaluation of antioxidant activity.

Determination of total antioxidant activity

The total antioxidant activity of FCLH, FCLR, ECLH and ECLR was evaluated as per method described by Chandini et al. (2008). Briefly 0.3 ml of sample was combined with 3 ml reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95 °C for 90 min. The absorbance of the solution was measured at 695 nm using a spectrophotometer (Shimadzu, UV-150-02) against blank after cooling to room temperature. The antioxidant activity is expressed as the number of equivalents of ascorbic acid in micrograms per gram of sample.

DPPH radical-scavenging activity

The scavenging effect of samples for DPPH radical were monitored according to the method described by Chandini et al. (2008). Briefly, 2.0 ml of test sample (in methanol) was added 2.0 ml of 0.16 mM DPPH methanolic solution. The mixture was vortexed for 1 min and then left to stand at room temperature for 30 min in the dark, and its absorbance was read at 517 nm using a spectrophotometer (Shimadzu, UV-150-02). The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{Scavenging effect (\%)} = \left[1 - \frac{(A_{\text{sample}} - A_{\text{sample blank}})}{A_{\text{control}}} \right] \times 100$$

Whereas A_{control} is the absorbance of the control (DPPH solution without sample), A_{sample} is the absorbance of the test sample (DPPH solution plus test sample) and $A_{\text{sample blank}}$ is the absorbance of the sample only (sample without DPPH solution). Synthetic antioxidant, TBHQ was used as a positive control.

Determination of reducing power

Reducing power was determined by method as described by Chandini et al. (2008). Briefly, 1.0 ml of different concentration (20, 40, 60, 80 and 100 µg) of sample was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1 %). Reaction mixture was incubated at 50 °C for 20 min. After incubation, 2.5 ml of trichloroacetic acid (10 %) was added and centrifuged (6500 g) for 10 min. From the upper layer, 2.5 ml solution was mixed with 2.5 ml distilled water and 0.5 ml FeCl_3 (0.1 %). Absorbance of all the sample solutions was measured at 700 nm using a spectrophotometer (Shimadzu, UV-150-02). Change in absorbance indicated reducing power with increased absorbance indicating increased reducing power.

ABTS radical scavenging activity

ABTS radical scavenging activity of samples was carried out by method mentioned by Sachindra and Bhaskar (2007a). ABTS radical solution was prepared by mixing 5 ml of ready to use ABTS solution with 100 ml acetate buffer (0.05 M, pH 4.5) and 5 units of peroxidase and incubating for 15 h at 37 °C. ABTS (1.9 ml) was mixed with 0.1 ml sample and incubated at 37 °C for 1 h. Control was prepared by adding 0.1 ml of distilled water instead of sample. For sample blank buffer was added instead of ABTS. Absorbance of the reaction mixture was observed at 734 nm by using a spectrophotometer (Shimadzu, UV-150-02). Scavenging activity was computed as follows.

$$\text{Scavenging (\%)} = \left[1 - \frac{(A_{\text{sample}} - A_{\text{sample blank}})}{A_{\text{control}}} \right] \times 100$$

Superoxide scavenging activity

Superoxide anion scavenging activity was determined by measuring the inhibition of the auto-oxidation of pyrogallol using by method described by Heo et al. (2005). Briefly, 0.3 ml of sample solution and 2.6 ml of 50 mM phosphate buffer (pH 8.24) were added into freshly prepared 90 μ l of 3 mM pyrogallol (dissolved in 10 mM HCl). Sample replaced with 0.3 ml of distilled water served as the control. The inhibition rate of pyrogallol auto-oxidation was measured at 325 nm. Absorbance of each sample was recorded at starting time of incubation (1 min) and at the end of incubation (10 min), increment of the absorbance was calculated by the difference of absorbance at 10 min - absorbance at the starting time.

Antibacterial properties

Antibacterial activity of samples was assessed as per agar well diffusion method described by Geis et al. (1983). The protein hydrolysates (FCLH & ECLH) and residue (FCLR & ECLR) were dissolved in distilled water at a concentration of 100 μ g/ml, and sterilized by filtration through a membrane filter (0.22 μ). Pre poured agar (Nutrient agar) media plates were overlaid with BHI soft agar and inoculated with freshly grown pathogenic bacteria. The pathogenic strains used include, *Listeria monocytogenes* MTCC657, *Bacillus cereus* F4433, *Staphylococcus aureus* MTCC1430, *Micrococcus luteus* MTCC2452, *Escherichia coli* MTCC118 and *Yersinia enterocolitica* MTCC8590. In agar plates, wells of 5 mm dia were made using a sterile cork borer. The wells were filled with 100 μ l of each of the samples and incubated for 18–24 h at 37 °C. After the incubation, the plates were examined for antagonistic zones around the individual wells and zone of inhibition (diameter) was measured in mm.

Statistical analysis

All the experimental results obtained were subjected to statistical analysis using statistica 1999 (Statsoft, OK, USA). Mean separation, wherever significant, was done using Duncan's multiple range test.

Results and discussion

Chemical composition

Proximate composition of chicken liver at various steps of fermentation and enzyme hydrolysis was performed and tabulated in Table 1. Crude protein content of raw chicken liver (RL) was recorded to 75.90 % on dry weight basis indicating that it could be a good source to produce protein hydrolysates.

In the present study minced and cooked liver was used as a substrate for fermentation and enzyme hydrolysis yielded 73.92 % on dry weight basis which was almost same as crude protein content of RL. Fermentative and enzymatically prepared chicken liver hydrolysates (FCLH & ECLH) had appreciable amounts of protein - 55.85 and 61.34 %, respectively, on dry weight basis. Residue left after fermentation and enzymatic hydrolysis (FCLR & ECLR) also had a considerable amount of protein which is presented in Table 1. FCLH and ECLH showed lower lipid (fat) content (5.29 and 5.23 %) as compared to that in unhydrolysed (18.91 %) and/or minced and cooked chicken liver (19.11 %). In general, during the hydrolysis process, structural lipids of the cell membrane are removed from the cells due to the formation of insoluble vesicles (Shahidi et al. 1995). Protein hydrolysates with low lipid content might significantly enhance the oxidative stability of the product thereby contributing towards shelf stability. FCLH and ECH had an ash content of 12.36 % and 10.75 % respectively. Increase in the ash content in FCLH may be due to salt (NaCl) added during the fermentation process.

pH, TTA and DH

The changes in pH and total titratable acidity (TTA) during fermentation are depicted in Fig. 2. The initial pH of minced and cooked liver was 6.13 and reduced to around 4.2 by the 24th hour of fermentation. With a reduction in pH, TTA increased from 465.7 μ l to 1114.6 μ l and corresponded with the reduction in pH. Increased TTA value indicates production of acids that are involved in hydrolysis. Fermentation process resulted in 14.3 ± 0.45 and enzymatic hydrolysis yielded 26.12 ± 0.59 ° of protein hydrolysis (DH). Results indicated that cleavage of peptide bonds was higher in enzymatic hydrolysis and led to the formation of smaller peptides and free amino acids. In one of the distantly related previous report, better emulsification and emulsion stability was observed even when the DH was low in case of salmon byproduct hydrolysate (Kristinsson and Rasco 2000). The reports concluded that hydrolysates prepared with Alcalase at low DH showed good foaming properties (Kristinsson and Rasco 2000; Gbogouri et al. 2004).

Antioxidant properties of FCLH & ECLH

Total antioxidant activity

The total antioxidant activity was evaluated by the phosphomolybdenum method based on the reduction of Mo(VI) to Mo(V) by the antioxidant compound and the formation of a green phosphate/ Mo(V) complex with a maximal absorption at 695 nm. The total antioxidant capacity of FCLH, assessed at 6th and 24th hour of fermentation and expressed as μ g AAE/mg protein are given in Table 2. The study reveals

Table 1 Proximate composition of raw chicken liver, fermented and enzymatic hydrolysed chicken liver ($n=6$; \pm SD)

Constituent, %	RL	MCL	LM		CLH		CLR	
			F	E	F	E	F	E
Moisture	75.72 \pm 0.65	76.70 \pm 0.93	72.25 \pm 0.50	75.49 \pm 7.36	5.48 \pm 0.70 ^a	3.92 \pm 1.05 ^b	3.93 \pm 0.70 ^p	3.37 \pm 0.13 ^p
Protein*	75.90 \pm 1.77	73.92 \pm 1.73	55.48 \pm 1.47	58.09 \pm 3.18	55.85 \pm 1.51 ^a	61.34 \pm 0.30 ^b	45.74 \pm 1.44 ^p	46.42 \pm 2.19 ^p
Fat*	18.91 \pm 0.40	19.11 \pm 0.47	18.49 \pm 0.88	18.66 \pm 5.52	5.29 \pm 0.80 ^a	5.23 \pm 2.10 ^b	14.80 \pm 0.60 ^p	14.62 \pm 2.34 ^q
Ash*	4.23 \pm 1.55	3.795 \pm 0.03	4.925 \pm 0.07	6.78 \pm 3.07	12.36 \pm 0.18 ^a	10.75 \pm 0.86 ^b	5.46 \pm 0.18 ^p	4.71 \pm 0.16 ^q

* : values are on dry weight basis; RL – Raw chicken liver; MCL – Minced and cooked liver; LM – Liver mass as such; CLH – Chicken liver hydrolysate; CLR – Chicken liver residue; F – Fermented; E – Enzymatic; SD – Standard deviation

^a & ^b : Row wise values amongst CLH carrying different superscripts are significantly different ($p<0.05$)

^p & ^q : Row wise values amongst CLR carrying different superscripts are significantly different ($p<0.05$)

that the antioxidant activity of the FCLH was $0.97\pm 0.07\%$ at 6th hour of fermentation and was $0.99\pm 0.02\%$ at 24th hour. The corresponding increase in protein content was from 31.30 ± 3.50 to $47.26\pm 2.88\%$. FCLR at 6th and 24th hour also exhibited noticeable total antioxidant activity - 0.49 ± 0.019 and $0.42\pm 0.09\%$, respectively. However, total antioxidant capacity of FCLH was double that of FCLR. Likewise, ECLH exhibited total antioxidant activity ($1.13\pm 0.17\%$) higher than that of ECLR ($0.28\pm 0.01\%$) as can be seen from Table 2. But, in all, the total antioxidant activity of ECLH was relatively higher than that observed in FCLH.

DPPH radical scavenging activity

The scavenging effects of samples for DPPH radical were monitored. The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. BHT and ascorbic acid are the reagents used as standards. Both FCLH & ECLH are able to reduce the stable radical DPPH to the yellow coloured diphenylpicrylhydrazine. The scavenging effect of FCLH at 6th and 24th hour on the

DPPH radicals is shown in Table 2. It is also revealed in Table 2 that there was no significant difference in DPPH radical scavenging at 6th and 24th hour (95.23 ± 0.55 and $96.14\pm 1.21\%$, respectively). FCLR also exhibited noticeable DPPH radical scavenging activity in the corresponding time (55.22 ± 0.66 and $75.07\pm 1.38\%$, respectively, at 6th and 24th hour). Results presented in Table 2 indicate that both ECLH ($92.76\pm 3.15\%$) and ECLR ($76.56\pm 2.61\%$) also exhibited DPPH radical scavenging, like their fermentation counterparts. Overall, FCLH relatively had more DPPH radical scavenging activity in comparison to ECLH. The hydrolysates in both the cases had better radical scavenging as compared to their respective residues. The EC_{50} (the effective concentration require to scavenge 50 % of the initial DPPH radicals) The EC_{50} value of FCLH and ECLH was 1.74 mg/mL and 1.28 mg/mL, respectively. Lower EC_{50} values indicate greater free radical scavenging capability and the results of this study compare well with that of chicken breast protein hydrolysate (Zhu et al. 2006) and wheat germ protein hydrolysate (Sun et al. 2012). During the hydrolysis process different types of low molecular weight peptides and amino acids are generated depending on the acid/enzyme specificity. Small size peptides and free amino acids affect the antioxidant activity (Wu et al. 2003). The results showed that both FCLH and ECLH could terminate the radical chain reaction by converting free radicals into more stable products. And, that they have smaller size peptides.

Determination reducing power

To measure reductive ability of the prepared hydrolysates, Fe^{3+} to Fe^{2+} transformation in the presence of FCLH & ECLH was investigated. Figure 3 clearly shows the reductive capability of FCLH at 6th and 24th hour as well as reducing power of ECLH. Both FCLH and ECLH exhibited dose dependent increase in the reducing power (Fig. 3). This corroborates well with the fact that increasing in the concentration of samples can increase reducing power (Sun et al. 2012). Amongst all the samples analysed, the FCLH exhibited the

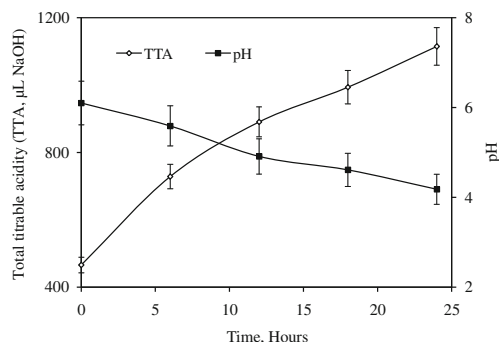


Fig. 2 Changes in pH and total titratable acidity (TTA) during fermentation of chicken liver with *P. acidilactici* NCIM5368. (Each value is expressed as mean of three determinations along with standard deviation)

Table 2 Various in-vitro antioxidant activities of chicken liver hydrolysates (FCLH & ECLH) and their residues (FCLR & ECLR). FCLH was analysed at 6th and 24th hour during fermentation ($n=6; \pm SD$)

Sample	Total antioxidant capacity* ($\mu\text{g AAE/mg protein}$)	DPPH radical scavenging*		ABTS radical scavenging*		Superoxide scavenging*	
		Inhibition %	EC ₅₀ (mg)	Inhibition %	EC ₅₀ (mg)	Inhibition %	EC ₅₀ (mg)
(a) Fermentatively obtaine chicken liver hydrolysate							
FCLH-6 h	0.97±0.07 ^a	95.23±0.55 ^a	1.64±0.01 ^a	19.16±0.64 ^a	9.17±0.27 ^a	64.87±2.3 ^a	2.47±0.085 ^a
FCLH-24 h	0.99±0.02 ^a	96.14±1.21 ^a	1.74±0.10 ^a	32.16±0.41 ^a	7.34±0.09 ^b	95.02±0.63 ^b	1.39±0.016 ^b
FCLR-6 h	0.49±0.019 ^c	55.22±0.66 ^c	2.23±0.03 ^c	10.66±3.74 ^c	11.53±4.31 ^c	16.33±6.14 ^c	5.95±3.05 ^c
FCLR-24 h	0.42±0.09 ^d	75.07±19.38 ^d	2.02±0.54 ^c	23.48±1.88 ^d	9.46±0.52 ^d	89.86±0.15 ^d	1.69±0.002 ^d
(b) Enzymatic chicken liver hydrolysate							
ECLH	1.13±0.17 ^p	92.76±3.15 ^p	1.28±0.01 ^p	19.29±0.17 ^p	8.10±0.78 ^p	88.94±0.55 ^p	1.85±0.32 ^p
ECLR	0.28±0.01 ^q	76.56±2.61 ^q	1.93±0.03 ^q	8.61±0.11 ^q	14.13±2.86 ^q	31.42±1.92 ^q	6.12±1.05 ^q

FCLH : Chicken liver hydrolysate obtained by fermentation; SD – Standard deviation

FCLH-6 h : FCLH at 6 h of fermentation; FCLH-24 h : FCLH at 24 h of fermentation

FCLR : Residue obtained upon separation of hydrolysate after fermentation

ECLR : Residue obtained upon separation of hydrolysate after enzymatic hydrolysis

^a & ^b : Column wise values amongst FCLH carrying different superscripts are significantly different ($p<0.05$)

^c & ^d : Column wise values amongst FCLR carrying different superscripts are significantly different ($p<0.05$)

^p & ^q : Column wise values amongst ECLH & ECLR carrying different superscripts are significantly different ($p<0.05$)

For DPPH, ABTS and superoxide scavenging activity 100 μg of FCLH or ECLH were dissolved in 1 ml

10 μg of lyophilized FCLH contains 5.5 μg protein while same amount of ECLH contains 6.1 μg protein

Antioxidant activity values for known antioxidant - 200 μl of α -Tocopherol (1 mg/ml) : DPPH – 86.56±0.001; ABTS – 99.03±0.024; Super Oxide – 100.8±0.05

highest reducing power. Fermentative and enzymatic hydrolysates had a higher reductive capability than their corresponding residue. It has been reported that hydrolysates with high reducing power show a great ability to donate electrons to form stable compounds and thereby interrupt the free-radical chain reactions (Xie et al. 2008). In this context, both FCLH and ECLH exhibit an excellent ability to offer electrons thereby contributing their antioxidant activity.

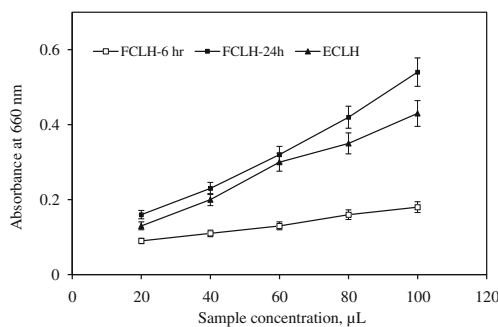


Fig. 3 Reducing power of different concentrations of chicken liver hydrolysates prepared by enzymatic hydrolysis (ECLH) and fermentation (FCLH). FCLH is shown at different stages of fermentation at 6 h (FCLH-6 h) and 24 h (FCLH-24 h). 100 μg of FCLH or ECLH were dissolved 1 mL and this stock solution was taken for assay. Each value is expressed as mean of three determinations along with standard deviation

ABTS radical scavenging activity

ABTS radical scavenging activity of both FCLH and ECLH was studied by monitoring the decay of the radical-cation produced by the oxidation of 2, 2-azinobis(3-ethylbenzothiaziline-6-sulfonate) (ABTS) caused by the addition of a phenolic-containing sample. ABTS radical scavenging activity (%) of FCLH and ECLH was comparatively lower than the results seen in case of other antioxidant assays. In case of FCLH, it ranged between 10.66±3.74 and 32.16±0.41 (Table 2). In comparison to FCLH, ECLH exhibited lesser activity (19.29 ±0.17 % or less) (Table 2). It should be noted that FCLH and ECLH had a DH of 14.3 and 26.12 %, respectively. This could have been the reason for relatively lesser scavenging activity by ECLH. It has been reported by an earlier work on hydrolysates from ornate threadfin bream muscle that higher DH (>20 %) results in decreased ABTS radical scavenging (Nalinanon et al. 2011).

Superoxide scavenging activity

The ability of FCLH and ECLH including their corresponding residues (FCLR & ECLR) to scavenge superoxide radical is presented in Table 2. Superoxide scavenging activity (%) of FCLH ranged from 64.87±2.3 to 95.02±0.63 depending on the stage of fermentation (6th hour or 24th hour) (Table 2).

Similarly, ECLH showed a scavenging activity (%) of 88.94 ± 0.55 (Table 2). From the results in Table 2, it can be noted that the superoxide radical scavenging activity of FCLH and FCLR, irrespective of the stage of fermentation, exhibited almost similar activity. However, ECLH had almost double the scavenging activity as that of ECLR (Table 2). Superoxide radicals are an extremely toxic radical species that are generated in various biological reactions and they are the precursor's for highly reactive species such as peroxide and hydroxyl radicals. Living cells have a competent biological defense mechanism in which enzymatic antioxidants involve in the conversion of reactive oxygen species/reactive nitrogen species (ROS/NOS) to harmless molecules. One such system is the conversion of superoxide anion to oxygen (O_2) and H_2O_2 by superoxide dismutase (SOD) (Huang et al. 2005). However, at times, excess production of superoxides may overwhelm the body's ability to scavenge these superoxide radicals. The superoxide radical scavenging activity of both type of hydrolysates (FCLH & ECLH) points to their potential to decrease the possible toxicity of superoxide radicals.

Antibacterial properties

The antibacterial activities of all samples (hydrolysate and residue) were evaluated against Gram-positive (*Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus* and *Micrococcus luteus*) and Gram-negative (*Escherichia coli* and *Yersinia enterocolitica*) bacteria by agar well diffusion method. The extent of inhibition zone (expressed in mm) indicated the antibacterial activity. FCLH was found to exhibit a strong antibacterial activity against *L. monocytogenes* (30 mm) and *B. cereus* (28 mm) and moderate inhibition of *M. luteus* (18 mm) and *Y. enterocolitica* (18 mm). ECLH showed moderate antibacterial activity against *M. luteus* (12 mm) and did not inhibit the others. The hydrolysates (FCLH and ECLH) showed better antibacterial activity than their residues (FCLR and ECLR) (data not shown). However, none of the samples inhibited *E. coli*. The results corroborate well with reports that state that Gram negative are more resistant than Gram positive bacteria (Lambert et al. 2001). Peptide fractions from different meat protein hydrolysates with low molecular weight range of 400 and 1400 Da exhibited the strongest antibacterial activity (Joseph et al. 2011). Further, generally, antibacterial property in fermented products could be either due to production of bacteriocins by lactic acid bacteria or formation of small peptides by hydrolysis of proteins. The antibacterial spectrum of FCLH did not correlate with that of *Pediococcus acidilactici* (Jini et al. 2011) used for fermentation. Thus, it can be concluded that the antibacterial activity of FCLH was more due to peptides present rather than the strain used in the fermentation process.

Conclusions

The chicken liver hydrolysates (FCLH & ECLH) have considerable amount of protein content (55.85 and 61.34 %) and exhibit both anti-oxidative and anti-bacterial characteristics. Both FCLH and ECLH scavenged close to 90 % of radicals except in case of ABTS radicals. FCLH showed excellent antibacterial activity whereas ECLH exhibited moderate activity. This innovative study thus proves that poultry byproducts could be converted into products with biofunctional activity which will have potential for use in the preparation of high protein foods and formulation of functional foods/nutraceuticals that can effectively be used for oxidative stress management.

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