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Effect of Hydrolysis Temperature on *in vitro* Bioaccessibility and Antioxidant Properties of Unicorn Leather Jacket (*Aluterus monoceros*) Skin Collagen Hydrolysates Following Simulated Gastro-Intestinal Digestion

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A B S T R A C T

The aim of the research was to prepare collagen hydrolysate (CH) from unicorn leatherjacket (*Aluterus monoceros*) fish skin using papain at different hydrolysis temperature viz. 5^0C (CH-5), 20^0C (CH-20), 25^0C (CH-25) and 50^0C (CH-50) and to investigate the antioxidant properties (DPPH free radical-scavenging activity, ferric reducing power assay, ferric chelating activity and hydroxyl radical scavenging activity) prior to and after *in vitro* gastrointestinal (pepsin–pancreatin) simulated digestion in order to assess the bioaccessibility and biofunctional activities. The DH was higher (4.27%) for CH-50 within 300 min of hydrolysis than other CH's. On contrary, ferric reducing and ferric chelating properties were comparatively higher in CH-5 than other CH's, corresponding to 33% and 0.32 absorbance units, respectively at 8 mg/mL protein concentration. DPPH free radical-scavenging and hydroxyl radical scavenging activities were more or less similar in all CH's. Subsequent *in vitro* gastrointestinal simulated digestion studies showed that the bioaccessibility of CH-50 was higher (54.25) after gastric and pancreatic digestion, but CH-5 exhibited good antioxidative property expressing 90% activity. FTIR spectral analysis further confirmed it through expression of major shifts in amide A, I and II peaks and disappearance of aromatic ring peaks in CH-5. The study thus indicated that CH with good antioxidant activity shall be produced by hydrolysis the skin at 5^0C, rather than at 50^0C.

Introduction

Collagen is the predominant protein of connective tissue in animals, making up about 30% of the total protein. It has a wide range of application in pharmaceutical and biomedical industries, which include tissue engineering for implants in humans, inhibition of angiogenic diseases, treatment of hypertension, urinary incontinence and osteoarthritis (Lee *et al.*, 2001). The main sources of industrial collagen are those from pig and bovine skin and bones. The outbreaks of Bovine Spongiform Encephalopathy, Foot

and Mouth disease and certain religious concern have resulted in causing restriction on animal collagen trade. On the other hand, marine collagen obtained from skins, bones, scales, as well as swim bladder of fish turned out as a better alternative. Collagen hydrolysate is a polypeptide made by hydrolysis of denatured collagen (Gomez-Guillen *et al.,* 2002). Collagen is enzymatically hydrolysed by a process employing commercially available proteolytic enzymes to liberate physiologically active peptides. By selection of suitable enzymes and controlling the conditions, the properties of the end product can be modified. Many studies have been conducted to extract and screen the potential bioactive properties of collagen hydrolysates from fish by products, such as gaint squid skin (*Dosidicus gigas*) (Alemán *et al.,* 2013), Croceine croaker scale (*Pseudosciaena crocea*) (Wang *et al.,* 2013), Spanish mackerel skin (*Scomberomorous niphonius*) (Chi *et al.,* 2014), and so on. Some collagen derived peptides exhibit good anti-oxidant activity, potent anti-hypertensive activity, anti-microbial activity against different strains of bacteria, protective effect on cartilage, or capacity to stimulate bone formation.

Unicorn leatherjacket (*Aluterus monoceros*) belonging to the order, Tetradontiformes and family, Monacanthidae, is a fish mainly used for fillet production and hence, large amounts of skin are discarded as wastes. The thick skin of the leatherjacket is a potential source for the production of fish collagen. In many of the previous studies, collagen hydrolysates are derived from the source raw material upon hydrolysis using suitable enzyme at 50^0C. It is known that fish collagen denatures to gelatin at 40-50^0C due to loss of their 3-dimensional structure. Formation of collagen hydrolysates at 50^0C is therefore expected to produce peptides with structural conformation similar to gelatin rather than collagen; exhibiting low biological functions. In order to understand the bioactive properties of collagen hydrolysates prepared at different hydrolysis temperature, this study was undertaken to examine their *in-vitro* antioxidant property prior to and after *in-vitro* gastro intestinal digestion.

Materials and Methods

Raw materials

Unicorn leatherjacket (*Aluterus monoceros*) belonging to the family, Monacanthidae is an important fishery in the East Coast of India. The flesh of the fish is edible, while the tough skin is discarded as wastes during fillet processing. Skins were procured from Sumaraj Seafoods Pvt Ltd, Mangalore, India; brought to the laboratory in iced condition and held at -20^0C until further analysis.

Extraction of collagen hydrolysates

Skins were thawed in runned water and chopped into small pieces prior to treatment with 0.8 N sodium chloride (NaCl) at a ratio of 1:6 (w/v) for 10 min at 5^0C to remove the impurities (Montero *et al.,* 1995). This process was repeated 3 times and the skins were washed again with cold distilled water. They were then treated with 0.1 N sodium hydroxide (NaOH) at a ratio of 1:10 for 3 days at 5^0C to remove the non-collagenous proteins and to prevent the effect of endogenous proteases on collagen (Sato *et al.,* 1986). The alkali solution was changed every day and the skins were washed with cold distilled water. They were then treated with 0.5 N acetic acid at a ratio of 1:6 for 30 min to cause swelling at 5^0C. The swollen skins (100 g) were homogenized with 200 mL of 50 mM phosphate buffer at a ratio of 1:2, for 5 min in a pestle and mortar. The pH of the homogenate was adjusted to 7.0 and 1% papain was added for hydrolysis. Hydrolysis

reaction was performed in a beaker placed in a magnetic stirrer at a constant agitation of 200 rpm at different incubation temperatures of 5^0C, 20^0C, 25^0C and 50^0C, to obtain collagen hydrolysates, CH-5, CH-20, CH-25 and CH-50, respectively.

Degree of hydrolysis

During the hydrolysis reaction, the degree of hydrolysis (DH) was determined periodically by the method described by (Adler-Nissen, 1979). DH is defined as the percentage of peptide bonds cleaved to determine the free amino group reaction with 2, 4, 6-trinitrobenzene sulfonic acid (TNBS). For the analysis, 0.25 mL aliquot was withdrawn at every 30 min time interval into 2 mL of 1% SDS and incubated at 75^0C for 15 min. From this, 0.25 mL of the sample was transferred into the test tubes containing 2 mL of 0.2 M sodium phosphate buffer (pH 8.2). A blank was prepared using 0.25 mL of 1% SDS. Then, 2 mL of TNBS reagent was added to all the test tubes, vortexed, covered with aluminum foil and incubated in dark at 50^0C for 50 min. The reaction was stopped by the addition of 4.0 mL of 0.1 N HCl and cooled at room temperature for 30 min. The absorbance was then read at 340 nm in a spectrophotometer. The amount of free amino group liberated was expressed as L-leucine equivalent as follows:

Degree of hydrolysis (DH) (%) = (Ct/Co) X 100

Where, Ct is free amino groups at time 't' and Co is total amino groups of samples.

On completion of hydrolysis for 360 min, the enzymes were inactivated by the addition of 6 M HCl. The hydrolysates were centrifuged at 13000 rpm in a refrigerated centrifuge (Eppendorf centrifuge 5804 R, Germany) at 4^0C for 10 min. The supernatant was collected as hydrolysates and neutralized to pH 7.0 using 1 N NaOH. The protein content of the collagen hydrolysates was estimated by the Biuret method using bovine serum albumin as standard.

DPPH free radical scavenging activity

DPPH free radical-scavenging activity was determined by the method described by (Yen and Wu, 1999). CH was diluted with distilled water to obtain a concentration of 1, 2, 3, 4 and 5 mg protein/mL. An aliquot of 1.5 mL of each CH was added to 1.5 mL of 0.1 mM DPPH in 99.5% ethanol and mixed thoroughly. The reaction mixture was held in a dark place for 30 min at room temperature. The absorbance was measured at 517 nm in a double beam UV–Vis spectrophotometer (Model V-530 Jasco, Japan). Distilled water was used as control.

DPPH free radical scavenging activity (%) = 1- (Abs sample / Abs control) ×100

Ferric reducing power assay

The ability of hydrolysates to reduce iron (III) to iron (II) was determined by the method described by (Oyaizu, 1986). An aliquot of 1 mL of each CH containing 1, 2, 3, 4 and 5 mg protein/mL concentration was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferric cyanide. The reaction mixture was incubated at 50^oC for 30 min and the reaction was stopped by the addition of 2.5 mL of 10% trichloroacetic acid (TCA). From that, 2.5 mL solution was pipetted out and mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$ solution. The solution was incubated for 10 min at room temperature and the absorbance was measured at 700 nm in a spectrophotometer. Distilled water was used as control. Higher absorbance of the reaction mixture indicated higher reducing power.

Ferric chelating ability

The ability of hydrolysates to chelate iron (II) was assessed by the method described by (Decker and Welch, 1990).

An aliquot of 1 mL of each CH containing 1, 2, 3, 4 and 5 mg protein/mL was first mixed with 3.7 mL of distilled water and then, 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine were added.

The reaction mixture was kept at room temperature for 20 min. The absorbance was measured at 562 nm in a spectrophotometer. Distilled water was used as control. Ferric chelating activity was calculated as follows:

Ferric chelating activity (%) = 1- (Abs sample/Abs control) ×100

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of hydrolysates was determined by the method described by (Smirnoff and Cumbes, 1989). An aliquot of 1 mL of each CH containing 1, 2, 3, 4 and 5 mg protein/mL was first mixed with 1.0 mL of 1.5 mM $FeSO_4$, 0.7 mL of 6 mM hydrogen peroxide and 0.3 mL of 20 mM sodium salicylate.

The reaction mixture was then incubated for 1 h at 37°C in a water bath. After incubation, the absorbance of the hydroxylated salicylate complex was measured at 562 nm in a spectrophotometer. Distilled water was used as control. The scavenging activity of hydroxyl radical was calculated as follows:

Hydroxyl radical scavenging activity (%) = 1- (Abs sample- Abs sample control) / Abs control) ×100

Where, Abs sample control is absorbance without sodium salicylate.

Attenuated total reflectance-Fourier transforms infrared spectroscopy (ATR-FTIR)

ATR-FTIR spectra of each CH as well as collagen were determined using an iD3 ATR-FTIR Spectrometer (iS5 NICOLET, Thermo Scientific, USA). KBr disks were first prepared with each sample in order to place onto the crystal cell and the cell was clamped into the mount of the spectrometer. The signal was collected from a range of 650-4000 cm^{-1} in 32 scans at a resolution of 4 cm^{-1} and was rationed against a background spectrum recorded from the clean empty cell at 25°C.

In vitro gastrointestinal (pepsin–pancreatin) simulated digestion

In vitro gastrointestinal stimulated digestion was determined to assess the physiological situation in the upper gastrointestinal tract (stomach and small intestine) based on the method described by (Gil-Izquierdo *et al.*, 2002) with slight modification. The simulated stomach solution was prepared with 3.2 g of pepsin, 2 g of NaCl and 7.0 mL of 12 N HCl and the final pH was adjusted to 2.0. Aliquot of 2.5 mL of each CH containing approximately 100 to 125 mg/mL protein was added to 20 mL of simulated stomach solution and the pH was adjusted to 2.0. The mixture was incubated at 37°C in a shaking water bath for 2 h. At the end of the post-gastric digestion, the mixture was immediately cooled in an ice bath and then, an aliquot of 5 mL was removed and taken for analysis of protein, degree of hydrolysis and antioxidant activity. A segment of cellulose dialysis tubing having a pore size of 2.4 nm (Himedia, Lab, Mumbai) containing 5 mL of 1 M $NaHCO_3$ solution was placed in the remaining portion of the mixture. Pancreatin solution (10 mL) was prepared using 40 mg of pancreatin, 250 mg of bile salts and the pH was adjusted to 7.5. From this, 4.5 mL was added to the

mixture and incubated at 37°C for 2 h. After the pancreatic digestion, the enzyme was inactivated by the addition of 6 N HCl until pH 2.0. The mixture was then centrifuged at 13000 rpm for 20 min at 4^0C and the supernatant was collected and neutralized to pH 7.0. The protein content, degree of hydrolysis and DPPH radical scavenging activity of the pepsin digest, pancreatin digest and dialyzed content were determined as described earlier.

Results and Discussion

Changes in the degree of hydrolysis of fish skin collagen

The DH of fish skin collagen varied at different reaction temperature. As shown in (Fig. 1), the percentage of DH of CH-50 increased to 4.27% and reached the maximum within 300 min of hydrolysis, while in CH-5 CH-20 and CH-25, the hydrolysis was very gradual in the beginning and later increased to 3.9-4.2%. The results indicated that the hydrolysis of collagen at higher temperature (50^0C) released more peptides than at lower temperatures. Several proteolytic enzymes can be employed for the hydrolysis of fish protein such as trypsin, pepsin, papain, alcalase, flavourzyme, and bromelain (Ghanbari *et al.,* 2012).

The enzyme, papain used for hydrolysis is a cysteine protease that breaks peptide bonds involving the use of a catalytic triad (His-Asn-Cys) with a deprotonated cysteine to cleave basic aminoacids (arginine and lysine) and hydrophobic aminoacids (leucine and glycine) (Paul and Leemor, 2007). The type of enzyme and substrate are known to influence the DH. Fan *et al.,* indicated that much higher DH of 13.8, 15.1 and 12.7% could be achieved using properase E, pepsin, trypsin and flavourenzyme, respectively than other proteolytic enzymes (Fan *et al.,* 2012). A maximum DH of 15% could be achieved with the commercial proteolytic enzymes of plant and animal origin. Microbial enzymes possess excellent activities on specific substrates and however their commercial production on large scale is still in a preliminary stage. As papain is readily available commercially at low cost, the same has been employed in this study to examine the DH of the unicorn leatherjacket fish skin.

Fish collagen, in general, possesses lower denaturation temperatures than that of animal collagen. The denaturation temperature of fish collagen varies with the fish species, their habitat and method of extraction (Muralidharan *et al.,* 2013). Tropical fish collagen possesses higher denaturation temperature (27-34^0C) than that of temperate fish collagen (16-20^0C) (Ogawa, 2004). As the unicorn leatherjacket fish is a tropical fish, the skin collagen from it had an average denaturation temperature of 28^0C (data not given). The collagen hydrolysates are produced through the breakage of corresponding peptide bonds in the 3-dimensional collagen network by specific proteases at their cleavage sites. With the increase in the hydrolysis temperature, the collagen strands begin to denature (uncoil) through breakage of intra-molecular and inter-molecular hydrogen bonds in the α-helix strands, and later hydrolyzed by the proteinases to yield much smaller peptides, as in the case of CH-50. Commercially, collagen hydrolysates are produced by treating the fish skin at higher temperatures (50^0C) followed by hydrolysis using proteases.

The application of low temperatures (5^0C-25^0C) for hydrolysis is therefore expected to yield collagen peptides with different conformation due to the inheritance of intra and inter molecular hydrogen bonds and other electrostatic interactions that provide more biofunctional properties.

Antioxidative activities of collagen hydrolysates

The antioxidative properties of CH prepared at four different hydrolysis temperatures are shown in (Fig. 2). The average DPPH radical scavenging activity of CH-5 was 69% at 12 mg/mL protein concentration ($p<0.05$) but those of CH-20, CH-25 and CH-50 were slightly lower with the corresponding increase in hydrolysis temperatures. Dose dependent activities were recorded with the increase in the concentration of all the hydrolysates from 2 to 14 mg/mL protein concentration. The relative IC_{50} values of unicorn leatherjacket fish skin collagen hydrolysates *viz*. CH-5, CH-20, CH-25 and CH-50 were recorded as 8, 7.5, 7.5 and 7 mg/mL, respectively. Earlier, (Fan *et al.,* 2012) have reported an IC_{50} value 1.92 mg/mL for tilapia frame protein hydrolysates probably because of the use of the trypsin enzyme, which had yielded different bioactive peptides.

The average ferric chelating activity in CH-5 was much higher than the other hydrolysates (Fig. 3). A maximum ferric chelating activity of 33% at 8 mg/mL protein concentration was recorded in CH-5.

The activity exhibited by CH-50 was lesser than that of other hydrolysates. Previous studies on the ferric chelating properties of CH are very limited. Fe^{2+} and Cu^{2+} are the two major metal pro-oxidants that induce oxidation. Metal chelating properties are examined to study the effect of biomolecules in chelating either of these two metal ions. A study that investigated the Cu^2 chelating activity of jellyfish collagen hydrolysates and their fractions indicated that fraction 2 having 3KDa molecular size exhibited the highest activity of 56.5% at 0.5 mg/mL; while those above >3KDa molecular size showed only 31.7% activity (Zhuang *et al.,* 2009). As the unicorn leatherjacket fish skin collagen hydrolysates contained peptides of varying molecular sizes, the Fe^{2+} chelating activity expressed was quite low. The relative IC_{50} values of CH-5, CH-20, CH-25 and CH-50 for Fe^{2+} chelating activity were recorded as 5, 4, 4, and 3.5 mg/mL, respectively.

The ferric reducing ability of plasma (FRAP) of CH-5 showed good activity with an average absorbance value of 0.32 at 8 mg/mL protein concentration (Fig. 4) than that of other hydrolysates. Temperature dependent activities were recorded for DPPH radical scavenging, ferric chelating and ferric reducing property of the CH.

The role of different proteinases enzymes in expressing the reducing ability of the resultant hydrolysates is therefore different. Papain produces hydrolysates with higher ferric reducing ability than bromelain. The relative IC_{50} values of CH-5, CH-20, CH-25 and CH-50 for the ferric reducing ability of plasma were 5, 4.5, 4.5 and 4.5 mg/mL, respectively.

The average hydroxyl radical scavenging activities of the CH were more or less similar, irrespective of the reaction temperatures (Fig. 5). At the protein concentration of 10 mg/mL, the average activities recorded were around 59%. Only at higher protein concentration, there was a slight decrease in the activities of CH-50 as compared to other hydrolysates. The relative IC_{50} values of CH-5, CH-20, CH-25 and CH-50 were recorded as 10, 7, 7 and 6.5 mg/mL, respectively. Fan *et al.*, reported that tilapia frame protein was hydrolyzed by different proteases, including properase E, pepsin, trypsin, flavourzyme, neutrase, gc106 and papain, to obtain three series of peptides (TFPH1, TFPH 2 and TFPH 3) by ultrafiltration through molecular weight cut-off membranes of 5, 3 and 1 kDa, respectively; and TFPH1 showed the highest hydroxyl radical scavenging activities with an IC_{50} value of 0.98 mg/mL (Fan *et al.,* 2012).

Attenuated total reflectance-fourier transforms infrared spectroscopy (ATR-FTIR)

In order to examine the difference in the structural conformation, the FTIR spectra of the different CH were analysed as shown in (Fig. 6). Fish collagen typically possessed five peaks *viz*. amide A (3431 cm^{-1}), amide B (2923 cm^{-1}), amide I (1641 cm^{-1}), amide II (1549 cm^{-1}) and amide III 1240 (cm^{-1}) due to their helical arrangements as reported earlier (Plepis *et al.,* 1996; Zhang *et al.,* 2014). As for CH, there were minor changes after hydrolysis in the positions of peaks. Amide B and amide III peaks were not detected in any of the CH obtained after papain digestion. This indicates that part of the collagen helical arrangements was destroyed or might be remaining in the unhydrolysed fraction.

Disappearance of amide III and a peak at 1456 cm^{-1} is an indication of the disappearance of triple helical structure (Plepis *et al.,* 1996). Amide III peak represents the combination peak between C-N stretching and N-H deformation from amide linkage as well as absorption arising from wagging vibrations from CH_2 groups of the glycine backbone and protein side changes. The peak at 1454 cm^{-1} corresponds to the existence of a CH bending vibration. The disappearance of helical structure after pepsin and trypsin hydrolysis of collagen was also reported earlier by few workers (Chi *et al.,* 2014).

Among the CH, no major modifications were noticed in amide A (3432 cm^{-1}) except that a small shift was observed in CH-5 (3426 cm^{-1}). The peak at the wave number 2360 cm^{-1} remained unchanged in all the hydrolysates, while the peak at 2341 cm^{-1} was not detected in CH-5. The peak at 2341 cm^{-1} was unique in CH, except CH-5 mainly corresponding to the C-H vibrations due to triple bonds. A peak at 2245 cm^{-1} was noticed only in CH-50. In respect of amide I peak corresponding to C=O stretching, there was a positive shift noticed only in CH-5 (1644 cm^{-1}). Amide II peak at 1560 cm^{-1} corresponding to C-N stretching and N-H bending also exerted a small shift in CH-5 and CH-25. The peak at 1412 cm^{-1} also showed minor changes in CH-5 and CH-25. Another peak at 1339 cm^{-1} indicating the presence of nitro compounds (N-O) showed a shift in CH-5 (1343 cm^{-1}). The peaks corresponding to the presence of alcohol, ether, ester etc with C-O were noticed in all the hydrolysates.

Fig.1 Degree of hydrolysis of collagen hydrolysates of unicorn leatherjacket skin prepared using papain enzyme

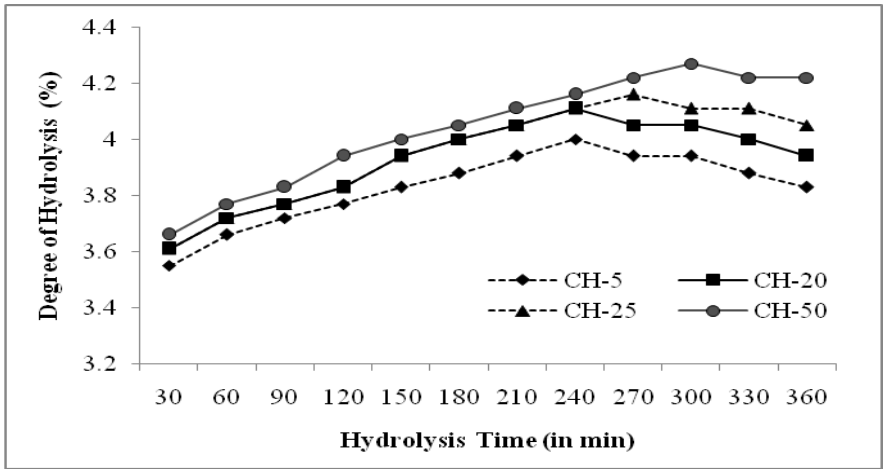


Fig.2 DPPH radical scavenging activities of unicorn leatherjacket skin collagen hydrolysates

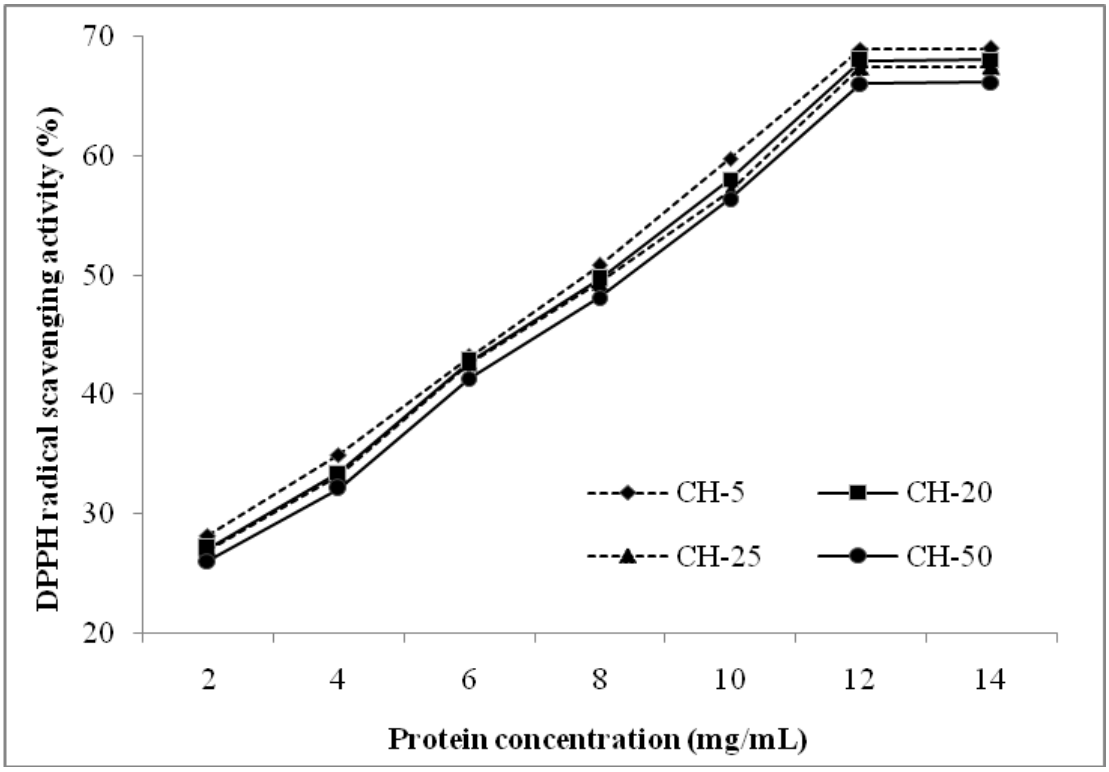


Fig.3 Ferric chelating activities of unicorn leatherjacket skin collagen hydrolysates

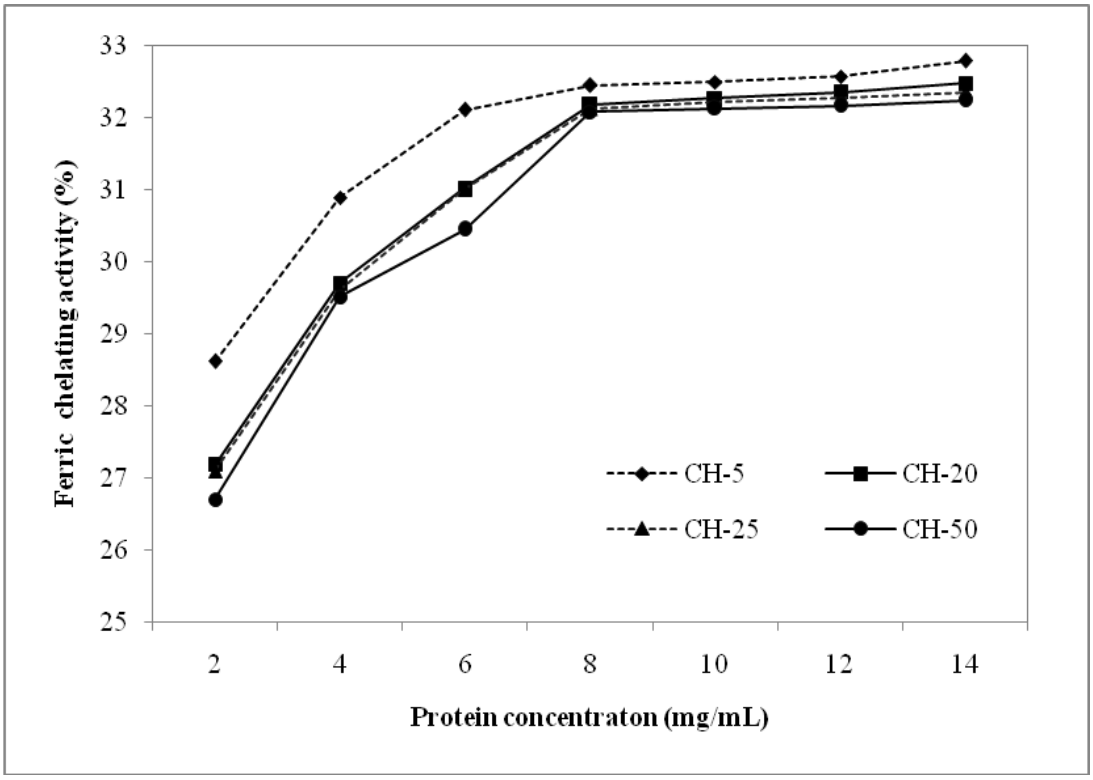


Fig.4 Ferric reducing ability of unicorn leatherjacket skin collagen hydrolysates

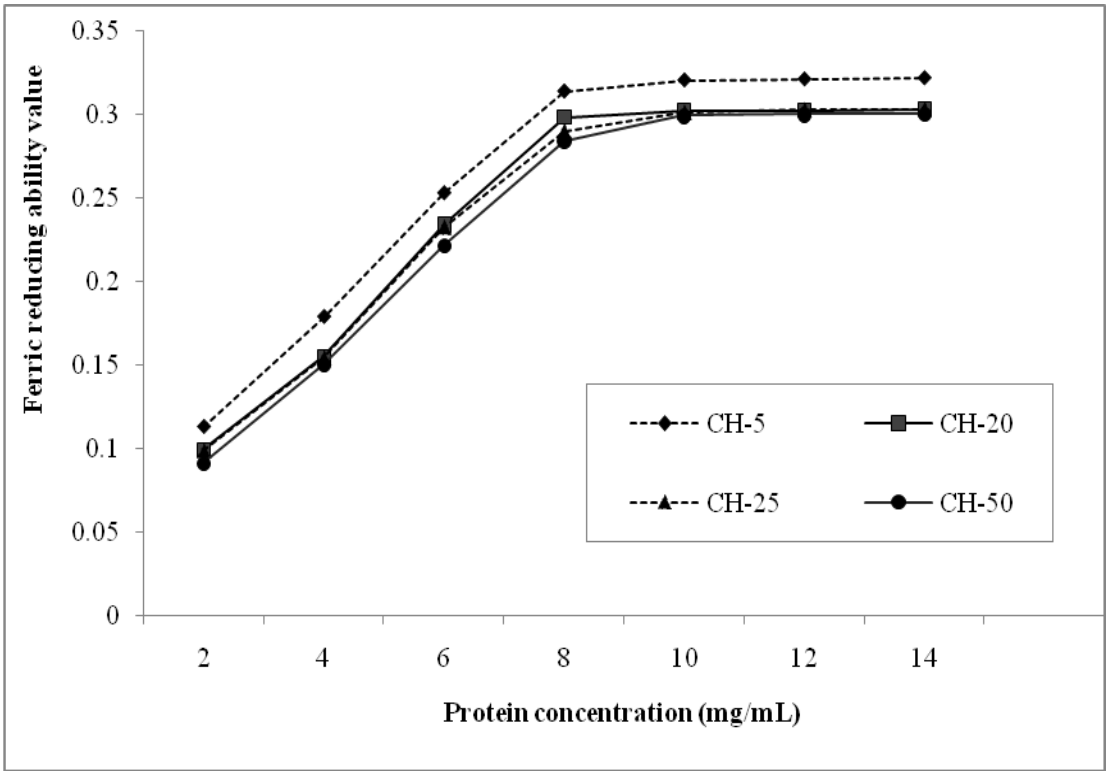


Fig.5 Hydroxy radical scavenging activities of unicorn leatherjacket skin collagen hydrolysates

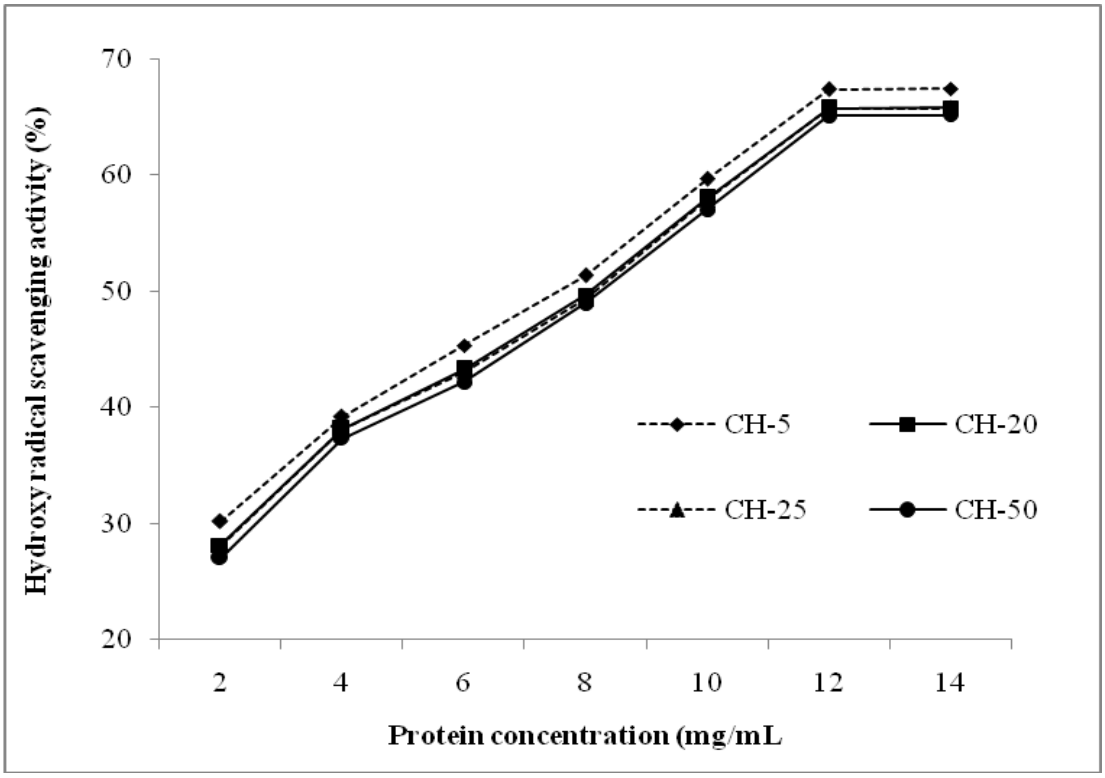


Fig.6 Attenuated total reflectance-Fourier transforms infrared spectroscopy of collagen and collagen hydrolysates of unicorn leatherjacket skin

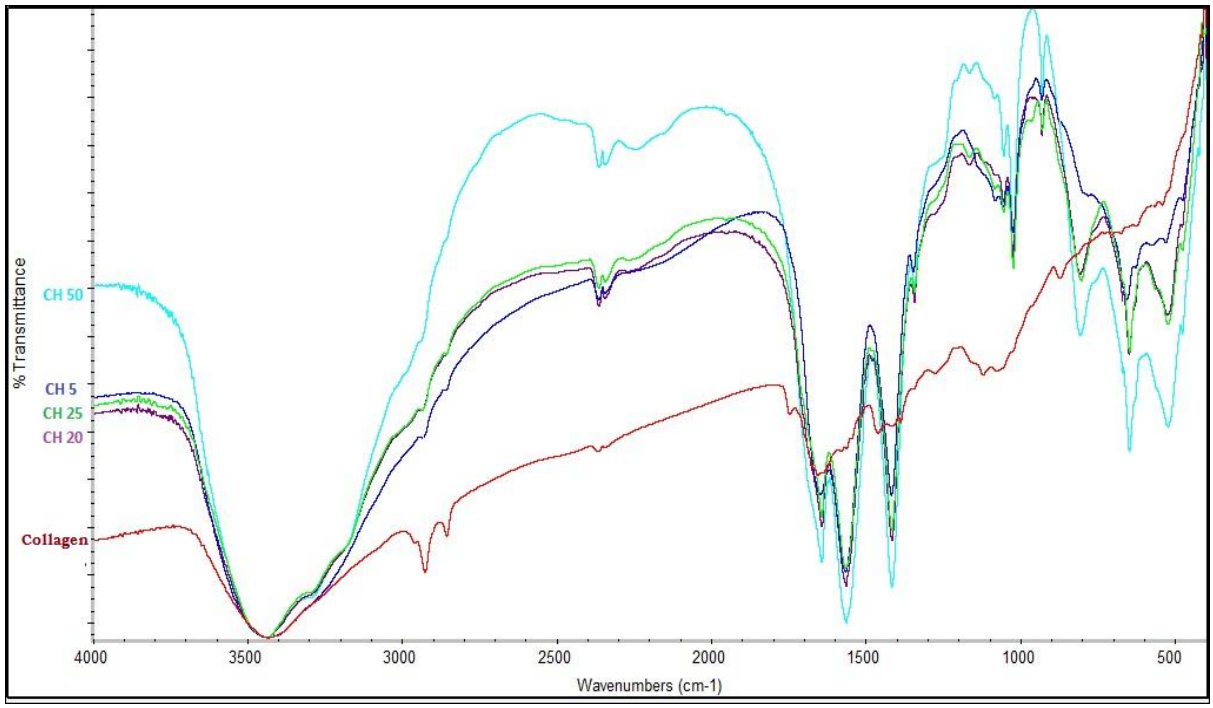


Fig.7 *In-vitro* gastro intestinal digestion of unicorn leatherjacket skin collagen hydrolysates

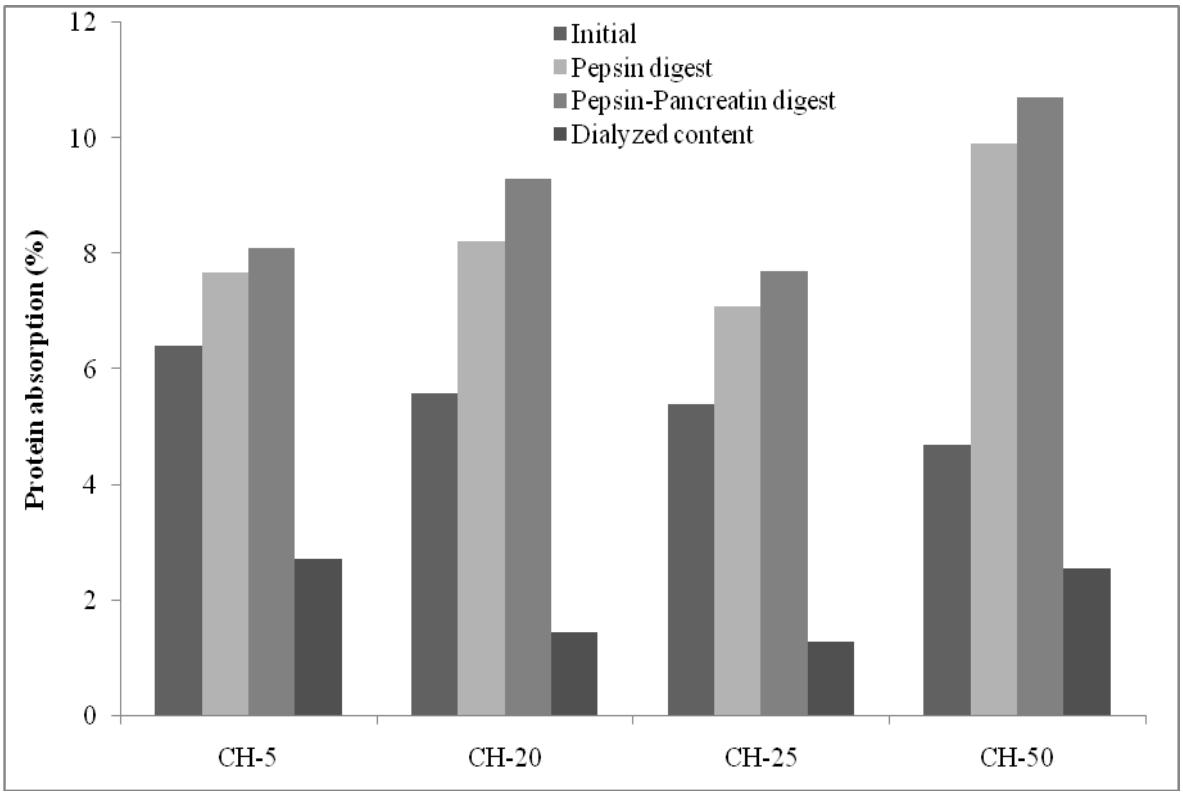


Fig.8 Degree of hydrolysis of *in-vitro* gastro intestinal digestion of unicorn leatherjacket skin collagen hydrolysates

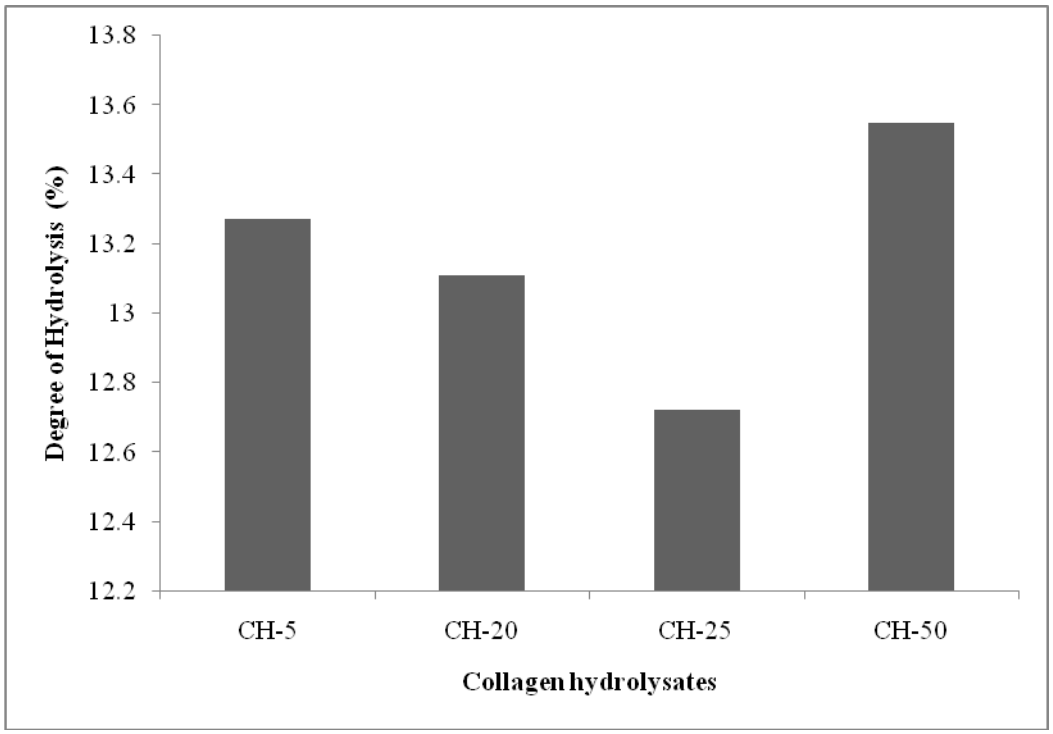
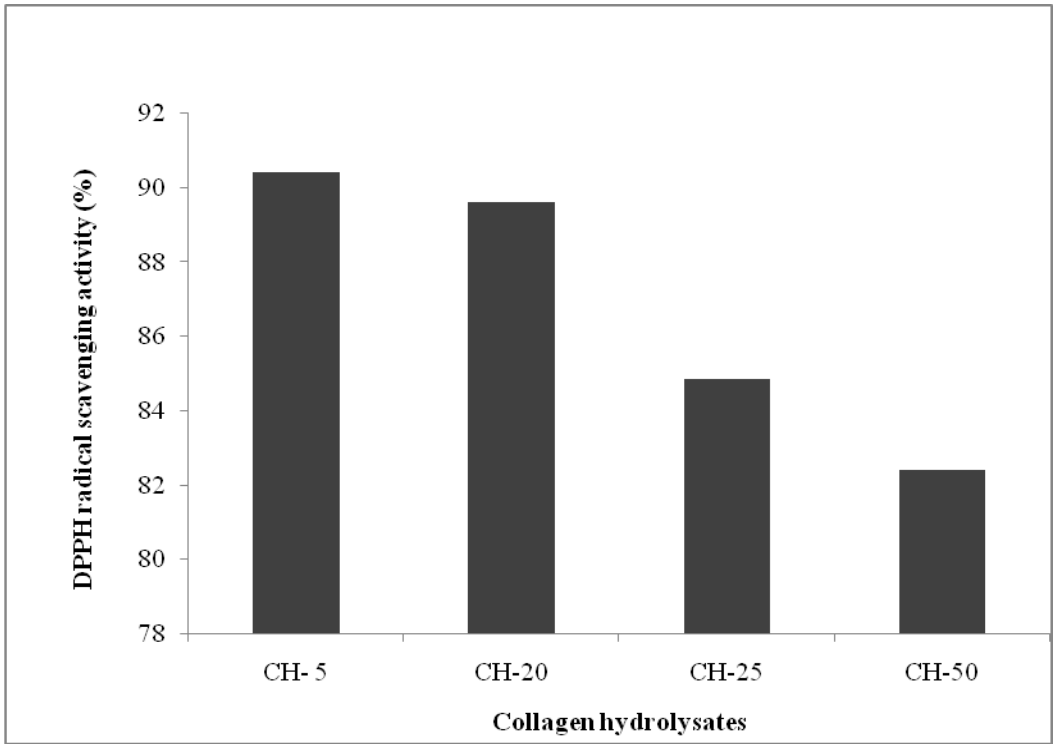


Fig.9 DPPH radical scavenging activity of *in-vitro* gastro intestinal digestion of unicorn leatherjacket skin collagen hydrolysates



Presence of aromatic rings as indicated by C-H peaks was detected at 803 cm^{-1} in all the hydrolysates but not in CH-5. Similarly, bromoalkanes (C-Br) peak at wavenumber 520 cm^{-1} was not present in CH-5 and CH-20; however, chloroalkanes (C-Cl) peak at 645 cm^{-1} was found in all the hydrolysates. The shift noticed in many of the functional groups of CH-5 distinguished it from other hydrolysates. Correspondingly, the presence of an additional peak at 2245 cm^{-1} is typical for CH-50 indicating their derivatization from gelatin and not collagen. This peak corresponds to C=C alkanes as well as C=N nitriles groups in the respective molecule.

In-vitro gastro intestinal digestion of collagen hydrolysates

Changes in the protein concentration of pepsin and pepsin-pancreatin digests; as well as dialysed extract following *in-vitro* gastrointestinal digestion of CH are shown in (Fig. 7). The protein concentration of CH varied between 4.7 to 6.4 mg/mL in the digestion solution. It increased in the pepsin digest of the hydrolysates due to the incorporation of enzymes, pepsin and pancreatin proportionately in the digestion solution. The absorption of the protein through the dialysis membrane was also estimated in the dialyzed content to examine the rate of absorption of peptides. As shown in Figure 6, the rate of peptide absorption of the CH was significantly high in CH-50 (54.25%) after gastric and pancreatin digestion. The average rate of absorption of collagen peptides from CH-5 was 42.5%, while from CH-20 and CH-25, it was only 25.89% and 24.07%, respectively. As the temperature increased, the rate of absorption subjected to gastric and pancreatic digestion decreased in the collagen peptides hydrolysed at low temperatures. Collagen being a connective tissue protein is not completely digested by the gastric and pancreatin enzymes; and hence the absorption of collagen peptides was quite limited. The higher rate of absorption of CH-50 peptides was probably because of the partial denaturation of intact collagen strands into gelatin at 50^0C and subsequent hydrolysis by proteinases yielding more denatured form of peptides, as noticed through IR spectral analysis.

Degree of hydrolysis of *in vitro* gastro intestinal digests

DH of the CH in the corresponding digests are shown in (Fig. 8). The average DH was significantly more in CH-50, CH-5, CH-20 and CH-25. After pepsin digestion, the average DH activities increased in CH-50, CH-5, CH-20, and CH-25 in accordance with the pepsin-induced hydrolysis. When the pepsin digest was further hydrolysed with pancreatin, the average DH further increased in all the hydrolysates. In order to examine the biological property of the absorbed peptides of the different collagen hydrolysates, the free amino groups in the dialyzed content was estimated. The free amino groups in the bioaccessible collagen peptides as compared to the pancreatin digest were calculated and the results are shown in Figure 8. The percentage absorption of collagen peptides ranged between 12-14%. The free amino groups absorption was higher for CH-50, followed by CH-5, CH-20 and CH-25.

DPPH radical scavenging activity of *in vitro* gastro intestinal digests

The DPPH radical scavenging activities of the CH in the corresponding digests are shown in (Fig. 9). The average DPPH radical scavenging activity was significantly higher in the CH-5, CH-20 and CH-25 than that of CH-50. After pepsin digestion, the average DPPH radical scavenging activities increased

in the order of CH-5, CH-20, CH-25 and CH-50. When the pepsin digest are further hydrolysed with pancreatin, the average DPPH radical scavenging activity increased only at a marginal rate. As the CH are derived using commercial papain, the gastric pancreatin digestion did not hydrolyze the substrate further, which could to the reason for the marginal increase in the protein as well as the antioxidant activity in the corresponding pancreatin digests.

In order to examine the biological property of the absorbed peptides of the different CH, the DPPH radical scavenging activity in the dialyzed content was estimated. Interestingly, it was noted that the average DPPH radical scavenging activities of the dialyzed content of CH decreased significantly than that of the pancreatin digests. The retention of the activity in the bioaccessible collagen peptides as compared to the pancreatin digest was calculated and the results are shown in Figure 8. The percentage retention of activity after the absorption of collagen peptides ranged between 82-90%. The percentage retention of activity was more for CH-5, followed by CH-20 and CH-25 and CH-50. Eventhough the rate of absorption of peptides was more in CH-50, the DPPH activity expressed was less.

The results indicated that although small proportion of CH-5 were bioaccessible, they exhibited good antioxidative property. The CH-50, on the other hand, was highly bioaccessible, but did not exhibit good antioxidative activity. This clearly emphasis that peptides present in the CH-5 possessed more bioactivity than those available in the CH-50. The bioactivity of the collagen peptides is more related to the retention of their 3-dimensional molecular conformation that determines the physical, chemical and biological activities of the molecules. FTIR spectral analysis corresponds with it showing shift to lower wave number in major amide A, amide I and amide II peaks as well as causing shift towards higher wave number in N-O nitroso compounds peak at 1340 cm^{-1} and C-Cl chloroalkane peak at 645 cm^{-1}. Disappearance of aromatic rings at 803 cm^{-1} and bromoalkanes peak at 519 cm^{-1} in CH-5 provided a different conformational structure possessing more bioactivity even though bioaccessibility was comparatively low.

Although there were no studies on the anti-oxidative activity on the assessment of fish collagen hydrolysates following *in-vitro* gastro intestinal digestion. Three different squid skin peptide fractions of different molecular sizes, *viz*. 10-3KDa, 3-1KDa and <1KDa obtained through hydrolysis by esperase enzyme for the ACE inhibitory activity following simulated *in vitro* gastrointestinal digestion and found that digestion with pepsin followed by pancreatin gave an increase in low molecular weight peptides contributing to high tissue affinity and slow elimination rate ACE inhibitory activity (2). Further study is however essential to isolate the small molecular weight collagen peptides following ultrafiltration and to examine their bioaccessibility after gastric and pancreatic digestion to ascertain their biological activity, which shall provide much more detailed information on the bioaccessibility of small collagen peptides following pepsin and pancreatin digestion; and subsequent biological activity.

The results indicated that the hydrolysis of unicorn leatherjacket skin at different temperatures using papain collagen hydrolysates with different antioxidant properties. Although the degree of hydrolysis was more at 50^0C, collagen hydrolysates produced at 5^0C gave marginally good antioxidant properties, particularly in terms of ferric reducing and ferric chelating properties. Subsequent study proved good bioaccessibility of collagen hydrolysates

produced at 50^0C, but those produced at 5^0C only expressed excellent bioactivity. Hence, it is recommended to produce collagen hydrolysates from fish skin wastes at low temperatures rather than at 50^0C to retain good biological properties.

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