Turk. J. Fish.& Aquat. Sci. 19(7), 551-560
http://doi.org/10.4194/1303-2712-v19_7_02

RESEARCH PAPER

Turkish Journal of FISHERIES and AQUATIC SCIENCES

In vitro Anti-Cancer, Anti-Diabetic, Anti-Inflammation and Wound Healing Properties of Collagen Peptides Derived from Unicorn Leatherjacket (*Aluterus Monoceros*) at Different Hydrolysis

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Article History
Received 31 March 2018
Accepted 25 June 2018
First Online 05 July 2018

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Keywords
Fish skin
Collagen peptides
MTT assay
α-amylase inhibition assay

Abstract

In this study, skin of unicorn leatherjacket (Aluterus monoceros) was hydrolyzed using collagenase at three different temperatures viz. 5°C, 25°C and 50°C, to obtain collagen hydrolysates. Collagen peptides of low molecular masses (<3kDa) viz. CP-5, CP-25 and CP-50 were obtained by sequential ultrafiltration with tangential flow filtration (TFF) system. The results indicated that CP-5 exhibited the maximum in vitro anti-cancer, anti-diabetic and wound healing activities than CP-25 and CP-50. In MTT assay, CP-5 inhibited the viability of COLO320 cancer cells upto 49.78% at 1 mg/mL concentration, while the inhibition was only 29.92% by CP-50. Anti-diabetic activity as assessed by α-amylase inhibition was 80.45% by CP-5 at 1 mg/mL concentration, as against 75.81% and 71.17% by CP-25 and CP-50, respectively. Collagen peptides, in general, exhibited very low anti-inflammatory property, which ranged between 3.27% to 4.01%. In vitro wound healing tested by scratch assay showed good migration of 3T3-L1 cells in CP-5 treated than CP-25 and CP-50 at 0.2 mg/mL concentration. The study thus proved that collagen peptides derived from fish skin at low hydrolysis temperatures (50C) exhibited more bio-active activities than those hydrolysed at high temperatures (500C); and shall be effectively utilized in various bio-medical applications.

Introduction

Collagen is the predominant connective tissue protein in animals, constituting about 30% of the total protein. It has a wide range of applications in pharmaceutical and biomedical industries, such as tissue engineering for implants in humans, inhibition of angiogenic diseases, treatment of hypertension, urinary incontinence and osteoarthritis (Lee, Singla, & Lee, 2001). The major sources of industrial collagen are mainly of animal origin, ie. from pig and bovine skin and bones. The outbreaks of animal diseases such as bovine spongiform encephalopathy (BSE), foot and mouth disease (FMD), etc and certain religious concern have imposed restriction on the use of animal collagen (Ri, Hideyuki, & Koretaro, 2007). So, marine collagen derived from skins, bones, scales, as well as swim bladder of fish has gained importance as a better alternative for terrestrial animal collagen.

Collagen is enzymatically hydrolysed using proteolytic enzymes to liberate physiologically active peptides known as collagen hydrolysates (Gomez-Guillen *et al*., 2002). Selection of suitable enzymes and control of hydrolysis conditions can modify the

bioactive properties of the hydrolysates. Collagen peptides have their molecular weights in the range of 0.5 to 25 kDa, as against the parent native collagen protein with 300 kDa (Moskowitz, 2000). Commercially, they are derived from the source by hydrolysis using suitable proteolytic enzyme at 50°C (Sovik & Rustad, 2006). Hydrolyzed collagen extraction from the scales of croaker fish (*Pseudotolitus elongatus*) at temperature ranging from 60°C to 90°C is reported (Olatunji & Denloye, 2017).

Collagen peptides derived from marine origin have shown good anti-oxidant, potent anti-hypertensive and anti-microbial activities as well as protective effect on cartilage and stimulation of bone formation (Cheung, Ng, & Wong, 2015). Several authors have examined the bioactive properties of collagen hydrolysates extracted from fish by-products, such as gaint squid skin (*Dosidicus gigas*) (Aleman, Gomez-Guillen, & Montero, 2013), Croceine croaker scale (*Pseudosciaena crocea*) (Wang *et al*., 2013), and Spanish mackerel skin (*Scomberomorous niphonius*) (Chi *et al*., 2014). As most of the studies on collagen peptides pertain to antioxidative and anti-hypertensive properties, this study was aimed to examine their anti-cancer, anti-diabetic and wound healing properties.

In vitro cell proliferation MTT assay is one of the most widely used assays for evaluating preliminary anti-cancer activity of both synthetic derivatives and natural products (McCauley, Zivanovic, & Skropeta, 2013). Very few studies are available on the anti-cancer properties of fish collagen peptides. The effect of collagen and collagen peptides derived from bluefin tuna abdominal skin was studied on HepG2 and HeLa cells using MTT assay by Han, Uzawa, Moriyama, and Kawamura (2011). The collagen peptides derived from milk fish (*Chanos Chanos*) was studies on HeLa and HCT-166 cells using MTT assay by Baehaki, Suhartono, Sukarno, Syah and Setyahadi (2016). The α-amylase activity is measured *in vitro* by hydrolysis of starch in presence of α-amylase. The reduced intensity of blue colour indicates the enzyme-induced hydrolysis of starch into monosaccharides. Very limited studies are available on the anti-diabetic effect of collagen peptides. Zhu, Peng, Liu, Zhang and Li (2010) have tested the oligopeptides obtained from skin of the salmon (*Oncorhynchus keta*) for possible anti-diabetic effects on rats by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay. *In vitro* anti-inflammatory activity is performed based the stability of HRBC (human red blood cell) membrane, which is similar to lysosomal membrane. Lysosomal enzymes released during inflammation produce a variety of disorders. *In vitro* anti-inflammation activities of glandular extracts of albacore tuna (*Thunnus alalunga*) by HRBC assay (Azeem *et al*., 2010) and that of the digested peptides derived from Chum salmon (*Oncorhynchus keta*) in RAW 264.7 macrophage cells by WST-1 assay (Saigusa, Nishizawa, Shimizu, & Saeki, 2015) have been reported.

Wound healing assay is generally performed using multiple cell types to study cell polarization, matrix remodeling, cell migration, and numerous other biological processes (Yue, Leung, Mak, & Wong, 2010). Topical skin collagen or gelatin treatment has been proven to be effective in accelerating wound healing (Hori *et al*., 2007). Zhang *et al*. (2011) investigated the effects of skin gelatin obtained from the chum salmon (*Oncorhynchus keta*) on defective wound repair in the skin of diabetic rats for 14 days by scratch assay and reported that wound closure occurred within 14 days along with reduction in inflammatory response. Hu, Yang, Zhou, Li and Hong (2017) have also performed scratch assay to examine wound healing effect of collagen peptides derived from tilapia fish skin (*Oreochromis niloticus*) and observed that HaCaT cells migration caused wound closure within 24 h.

Unicorn leatherjacket (*Aluterus monoceros*) belonging to the order, Tetradontiformes and family, Monacanthidae, is a fish mainly used for fillet production. The thick skin of the fish leads to generation of large amounts of wastes, that form potential sources for the production of fish collagen. It is well- known that fish collagen denatures to gelatin at 40-50°C loosing their 3-dimensional secondary and tertiary structure. So, collagen hydrolysates formed at 50°C produce peptides with structural conformation similar to gelatin rather than native collagen. It is therefore uncertain on the bioactive properties of collagen peptides formed below its denaturation temperature than those produced at 50°C. Hence, this study was undertaken to extract collagen peptides from unicorn leatherjacket skin at three hydrolysis temperatures and to examine their *in vitro* anti-cancer, anti-diabetic, anti-inflammation and wound healing properties.

Materials and Methods

Fish Skins

Unicorn leatherjacket fish (*Aluterus monoceros*) is a file fish of family, Monacanthidae inhabiting coral reefs and feeds on benthic organic debris consisting of seaweeds and algae. This species caught as by catch in trawls and often exported in deskined forms from India. These skins, which are normally discarded as wastes were obtained from M/s Sumaraj Seafoods Pvt Ltd, Mangalore, India in iced condition for the extraction of collagen peptides. The skins were washed in potable fresh water to clean the debris and then held frozen at -20°C.

Chemicals

Formazan, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT), hypotonicity

induced human red blood cells (HRBC), 1X PBS (phosphate buffered saline), α-amylase from porcine pancreas in solution (Duramyl 300™, 300U 1.08g/mL), acarbose, fetal bovine serum (FBS), 3,5-dinitrosalicylic (DNS) acid, crystal violet and dimethyl sulfoxide (DMSO) were obtained from M/s Sigma-Aldrich Inc., St.Louis, USA. Dulbecco's modified eagle's medium (DMEM) were obtained from Nissui Pharmaceuticals, Tokyo, Japan. COLO320 (ATCC® CCL-220.1™) and 3T3-L1 (ATCC® CL-173™) cells were purchased from American Type Culture Collection (ATCC), Manassas, USA. Starch indicator and ethanol were purchased from M/s Thermo Fisher Scientific, Pvt Ltd, UK. Cyclophosphamide, paraformaldehye and diclofenac were purchased from M/s India Mart, Mumbai, India.

Extraction of Collagen Hydrolysates

For the extraction of collagen hydrolysates, frozen unicorn leatherjacket skins were thawed in running water and chopped into small pieces. Skins (100 g) were treated with 0.8 N NaCl (1:6; w/v) for 10 min at 5°C to remove the impurities, and this process was repeated three times. Skins were then washed with cold distilled water and treated with 0.1 N NaOH (1:10, w/v) for 3 days at 5°C to remove the non-collagenous proteins and to prevent the effect of endogenous proteases on collagen. They were then washed with cold distilled water and treated with 0.5 N acetic acid at a ratio of 1:6 (w/v) for 30 min to cause swelling at 5°C. Swollen skins were homogenized with 200 mL of 50 mM phosphate buffer (pH 7.0) at a ratio of 1:2 (w/v) for 5 min. For the hydrolysis, 1% collagenase, derived as crude enzyme from fish fins in our earlier study was added and the mixture was placed in magnetic stirrer at a constant agitation of 200xg at three different incubation temperatures viz. 5°C, 25°C and 50°C. The degree of hydrolysis (DH) was determined periodically during the hydrolysis reaction by TNBS method (Adler-Nissen, 1979).

$$DH\ (\%) = \left(\frac{Ct}{Co}\right) \times 100$$

where, "Ct" is free amino groups at time 't' and "Co" is total amino groups of samples. On completion of hydrolysis, the enzymes were inactivated by addition of 4 mL of 6 N HCl and centrifuged at 9000xg for 10 min at 4°C. The supernatant was collected as collagen hydrolysates and neutralized to pH 7.0 using 1N NaOH and filtered through 0.2 µm millipore syringe filter and the collagen hydrolysates were designated as CP-5, CP-25 and CP-50, respectively.

Fractionation of Collagen Peptides

Collagen peptides were fractionated from collagen hydrolysates based on their molecular weight cutoffs (MWCOs) using tangential flow filtration (TFF) system (Model- 901-0025 Thermo Scientific., USA). The hydrolysate was first filtered using the ultra membrane filters having MWCO <30kDa to obtain 30kDa biopeptide fractions. This fraction was consequently filtered through the ultra membrane filters with MWCO 10kDa and 3kDa to obtain <10kDa and <3kDa biopeptide fractions, respectively. The collagen peptides of <3kDa are used for the examination of bioactive properties.

***In vitro* Anti-Cancer Assay**

MTT assay was performed to examine the cytotoxicity effect of collagen peptides on cancer cells (Mosmann, 1983). For the assay, COLO320 cells were maintained in DMEM supplemented with 10% FBS and incubated for 3-4 days at 37°C in a humidified atmosphere with 5% CO_2 (Thermo Fisher Scientific, Pvt Ltd, UK). The cells were then plated in 96 well flat bottom tissue culture plates at a density of approximately $1.2x10^4$ cells/well and allowed to attach overnight at 37°C. The medium was then discarded and the cells were incubated with different concentrations of each collagen peptide (0.25-1 mg/mL) for 24 h. After incubation, medium was discarded again and 100µl of fresh medium was added with 10µl of MTT (5 mg/mL) and incubated for 4 h. The medium was then discarded and 100µl of DMSO was added to dissolve the formazan crystals. The absorbance was read at 570 nm in a microtitre plate reader meter (Thermo Fisher Scientific, Pvt Ltd, UK). Cyclophosphamide was used as a positive control. Medium along with cells (untreated) served as a control.

Cell survival was calculated by the following formula:

$$\text{Viability}(\%) = \left(\frac{\text{Sample OD}}{\text{Control OD}}\right) \times 100$$

$$\text{Cytotoxicity } \% = 100 - \text{Viability } \%$$

***In vitro* Anti-Diabetic Assay**

The α-amylase inhibition assay was carried out to determine the anti-diabetic properties of collagen peptides based on the modified method of Mccue and Shetty (2004). For the assay, different concentrations of each collagen peptide (0.2 -1 mg/mL) and 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing porcine pancreatic *α*-amylase enzyme (0.5 mg/mL) were incubated for 10 min at 25°C. After incubation, 500 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to the reaction mixture. Subsequently, the reaction mixture was incubated for 10 min at 25°C, followed by addition of 1.0 mL of DNS. Finally, the reaction was stopped by incubation in boiling water for

5 min and cooled to room temperature. The reaction mixture was then diluted with 10 mL distilled water, and the absorbance was measured at 540 nm in a UV-Vis spectrophotometer (Model- V-530, Jasco, Corporation Inc, Japan). The mixture of all other reagents and the enzyme, except the test sample was used as a control and the results of *α*-amylase inhibition activity were expressed in terms of inhibition percentage. Acarbose (10 mg/1mL) was used as a positive control.

$$\text{Inhibition (\%)} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right) \times 100$$

***In vitro* anti-inflammation assay**

HRBC membrane stabilization assay was used to determine the anti-inflammation activity according to the method of Gandhidasan, Thamaraichelvan and Baburaj (1991). Different concentration of each collagen peptide (0.05-0.2 mg/mL) in 0.2 M phosphate buffer was taken, to which, 0.5 mL of 10% hypotonicity induced HRBC suspension and 0.5 ml of 0.25 % hyposaline were added; incubated for 30 min at 37°C and centrifuged at 9000xg for 20 min at 4°C. The haemoglobin content in the supernatant solution was estimated in a UV-Vis spectrophotometer at 560 nm. Diclofenac was used as standard. A control was prepared by distilled water, instead of hyposaline to produce 100% hemolysis without samples. The percentage of HRBC protection was calculated by using the following formula:

$$\text{Protection (\%)} = 100 - \left[\left(\frac{\text{Optical density of test sample}}{\text{Optical density of control}}\right) \times 100\right]$$

***In vitro* Wound Healing Assay**

In vitro scratch assay was performed to determine the wound healing based on the method of Hu *et al*. (2017) with slight modification. For the assay, 3T3-L1 cells were were grown in DMEM supplemented with 10% FBS for 24 h. The cells were seeded into 24-well tissue culture plate at a density of 0.05 x 10^6 and grown for 24 h until cell growth reached 70-80% confluence as a monolayer. Then, scratches were made using 1 ml pipette tips without changing the medium to remove the detached cells. The wells were again replenished with fresh medium along with each collagen peptide (0.2 mg/mL). Then, the cells were allowed to grow for another 48 h and washed twice using 1X PBS. The cells were fixed with 3.7% paraformaldehye for 30 min and then stained with 1% crystal violet in 2% ethanol for 30 min. The gap distance of the scratch was then measured using an ORCA-ER CCD camera (Nikon Eclipse TE200: Nikon, Tokyo, Japan) connected to the phase-contrast microscope (Olympus CX41, Olympus Corporation, Japan). The scratch closure rate is expressed in percentage by the following formula:

$$\text{Scratch closure rate (\%)} = \left(\frac{A_0 - A_t}{A_0}\right) \times 100\%$$

where A_0 is the scratch area at 0 h, and A_t is the scratch area at the designated time

Results and Discusssion

Hydrolysis of Fish Skin Collagen by Collagenase

Collagenase was used for the hydrolysis of unicorn leatherjacket fish skin at different temperatures. As shown in Figure 1, the DH increased linearly with the increase in reaction time reaching the saturation point at 300 min and thereafter showed a slight decline. The DH recorded was low for CH-5 (6.6%), while it was high

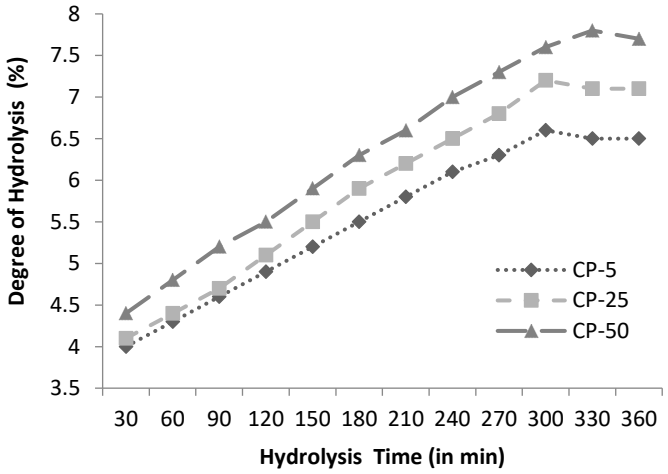

Figure 1. Degree of hydrolysis of collagen hydrolysates of unicorn leatherjacket fish skin.

for CH-50 (7.6%). Slightly high DH noticed in CH-50 was because of the high hydrolysis temperature, which had aided the release of more peptides. The type of enzyme, substrate and hydrolysis condition are known to influence the DH. In tilapia frame collagen peptides, the DH were recorded as 13.8, 15.1 and 12.7% using properase E, trypsin and flavourenzyme, respectively (Fan, He, Zhuang, & Sun, 2012). The relatively low DH recorded in leatherjacket skin collagen peptides was probably because of the use of crude collagenase. However, the collagenase derived from fish fins, generally otherwise discarded as wastes, shall be an effective alternative to commercial proteolytic enzymes.

Fish collagen, in general, possesses low denaturation temperatures as compared to animal collagen. Tropical fish collagen possesses slightly higher denaturation temperature (27-34°C) than temperate fish collagen (16-20°C) (Ogawa *et al*., 2004). As the unicorn leatherjacket fish is a tropical fish, collagen derived from its skin, it had an average denaturation temperature of 28°C (Muralidharan, Shakila, Sukumar, & Jeyasekaran, 2013). Performing hydrolysis beyond this temperature (50°C) using any proteolytic enzyme would denature (uncoil) the native structure of collagen by breaking the intra- and inter-molecular hydrogen bonds, electrostatic and hydrophobic interaction in the α-helix strands (Hassan *et al*., 2005); and at the same time proceed with breakdown of uncoiled collagen yielding more peptides. Collagen hydrolysates are commercially produced by treating the fish skin at 50°C by proteolytic enzymes. In this study we observed that, when low temperatures (5°C or 25°C) are employed for hydrolysis, although the DH was low, it yielded collagen peptides with better conformational structure retaining the native intra- and inter-molecular hydrogen bonds, hydrophobic and electrostatic interactions as well as disulfide bonds, which might help them to exhibit more biofunctional properties. The average yield of lyophilized collagen peptides obtained from leatherjacket skin ranged from 3.6 to 4.2% with an average protein content of 83%.

***In vitro* anti-Cancer Assay**

MTT assay was performed to examine the effect of collagen peptides on the growth, and viability of COLO320 cells. The cytotoxicity effect exhibited by collagen peptides ranged between 17-50% on COLO320 cells (Figure 2). The cell viability inhibition by CP-5 was relatively higher than that expressed by CP-25 and CP-50. The maximum activity exhibited by CP-5 was 49.78% at 1 mg/mL concentration, while those expressed by CP-25 and CP-50 were 32.67% and 29.92%, respectively. The positive control, cyclophosphamide expressed much higher activity of 75.74% at the same concentration. In a study conducted by Picot *et al.* (2006), fish peptides prepared using Protomex and alcalase exhibited 33.3% growth inhibition on HeLa cells, but 81.7% inhibition on HCT-166 cells at 1mg/mL concentration. In an another study, salmon and bluefin tuna skin collagen and collagen peptides exhibited the highest cytotoxicity activity of 65% and 50% at 0.2 mg/mL concentration against HepG2 and HeLa cells, respectively Han *et al*. (2011). In the same study, only bluefin tuna collagen reduced the growth of HepG2 and HeLa cells by 50% and 38%, respectively; but not the collagen peptides.

Similarly, the collagen peptides extracted from milk fish (*Chanos chanos*) exhibited of 70% and 18% inhibition in HeLa cells and HCT-166 cells at 1 mg/mL concentration, respectively Baehaki *et al*. (2016). Much

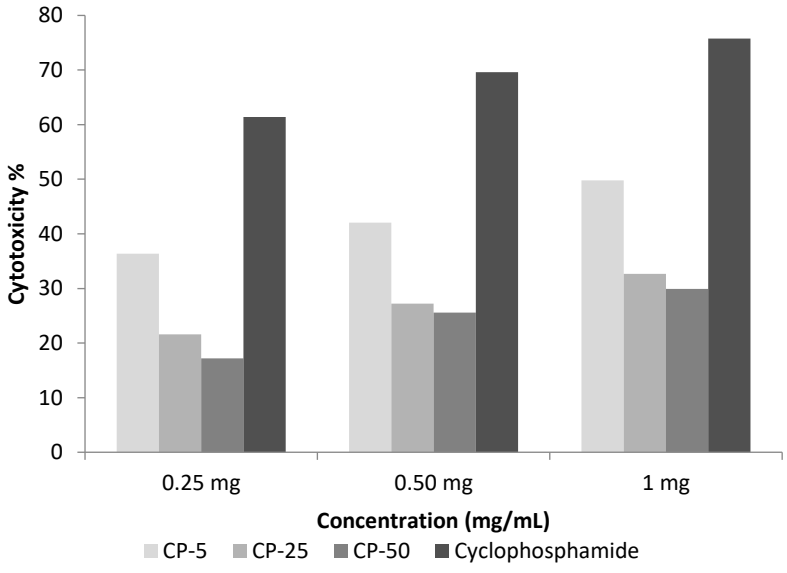

Figure 2. Cytotoxicity effect of collagen peptides on COLO320 by MTT assay.

lower cytotoxicity activity in HaCaT cells was recorded as 19.9±1.9% at 1 mg/mL concentration by fish scale collagen peptide (FSCP) (Subhan *et al*., 2017). It is clear from the above studies that reduction of cell growth by collagen or collagen peptides are affected by the type of the cell, type of the fish and even degree of hydrolysis (DH). Besides these studies, a 440.9Da anchovy peptide was found to induce apoptosis in human U937 lymphoma cells by increasing caspase-3 and caspase-8 activity (Lee, Kim, Lee, Kim, & Lee, 2003). Another hepcidin TH2-3 tilapia peptide (Chen, Lin, & Lin, 2009) and epinecidin-1 grouper peptide showed significant inhibition on human fibro sarcoma cells (Lin *et al*., 2009).

Inhibition of relative cell growth was most likely not due to cell apoptosis or necrosis; and the related mechanism seems very complex Subhan *et al*. (2017). As the hydrolysis of collagen induces collapse of the triple helix conformation, the obtained collagen peptides with different conformation influence the adhesion of molecules on the surfaces of different cancer cells. Hassan *et al*. (2005) have stated that shape and density of collagen strands have an effect on the growth of cell. Therefore, CP-5 that retains better conformational structure might have added functionally to interact with the selected cancer cell surface proteins cell growth causing inhibition than CP-25 and CP-50.

In vitro α-Amylase Inhibition Assay

Diabetes is usually caused by the interaction of genetic and environmental factors and is characterized by a lack of insulin secretion and insulin resistance, always leading to metabolic disorders of fat, protein and carbohydrates (Kumanan, Manimaran, Saleemulla, Dhanabal, & Nanjan, 2010). The α-amylases catalyzes the hydrolyses of α-1,4 glycosidic linkage carbohydrates, in the presence of calcium. In cases of excess of α-amylase activity and insulin deficiency, the level of blood glucose increases and causes type II diabetes Agarwal and Gupta (2016). The α-amylase inhibition assay was performed to examine *in vitro* anti-diabetic activity of collagen peptides. In this assay, the percentage inhibition of α-amylase increased in a concentration dependent manner of collagen peptides from 0.2 to 1 mg/mL concentration (Figure.3). The maximum inhibitions recorded were 80.45%, 75.81% and 71.17% for CP-5, CP-25 and CP-50, respectively at 1 mg/mL concentration. Acarbose, used as a standard drug, showed 98.35% inhibitions at the same concentration. The IC_{50} values of collagen peptides were calculated as 1.17, 1.92 and 2.65 mg/mL for CP-5, CP-25 and CP-50, respectively. Earlier, Fernandez, Moyano, Dıaz, and Martınez (2001) have reported that collagenous gut of red porgy (*Pagrus pagrus*), common pandora (*Pagellus erytrhinus*) *and* annular seabream (*Diplodus annularis*) gave the maximum inhibition of 30%, 65% and 61% α-amylase activity at 20 µl/mL concentration, respectively. The α-amylase inhibitions expressed by unicorn leatherjacket collagen peptides were more or less in accordance with the reported values. The fish protein hydrolysate extracted from crucian carp (*Carassius carassius*) had shown only 20.03±0.89% of α-amylase inhibition at 1.2 mg/mL concentration (Liu, Wang, Peng, & Wang, 2013), which indicated that fish muscle derived peptides exhibited less α-amylase inhibition than collagen derived peptides. The α-amylase inhibition thus varied with the substrate, peptide composition and even hydrolysis temperature.

In vitro Anti-Inflammatory Assay

Inflammation occurs in response to an infection due the action of innate immune cells such as

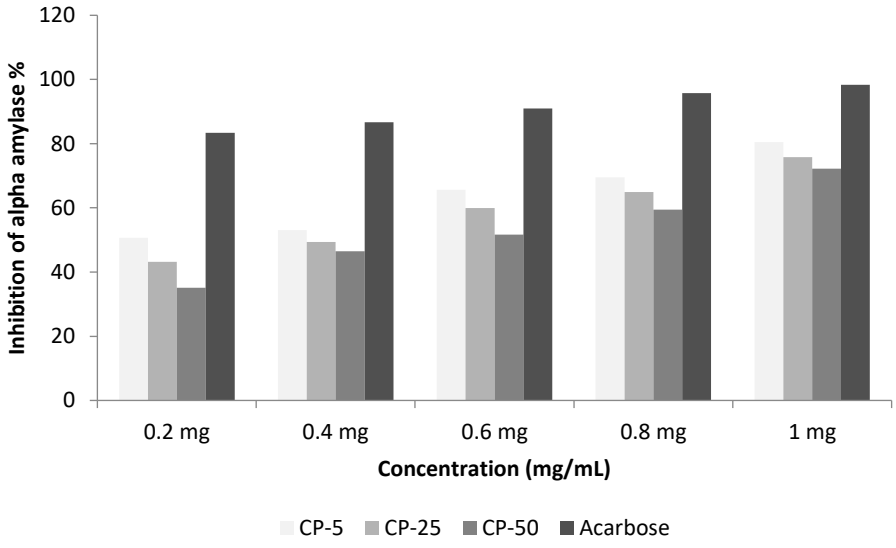

Figure 3. Percentage α-amylase inhibition of the collagen peptides.

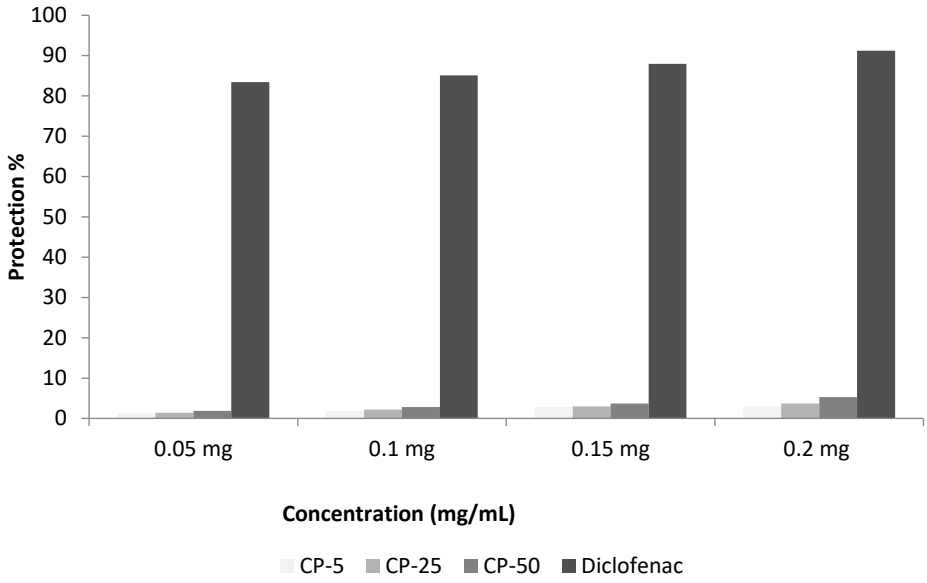

Figure 4. Anti-inflammatory percentage protection activity of collagen peptides.

macrophages and neutrophils, which produce various cytokines and chemokines. Prostaglandin is released extracellularly as inflammatory response. Anti-inflammatory drugs inhibits prostaglandin synthesis either through cyclooxygenase (COX) and lipoxygenase (LOX) inhibition or through stabilization of lysosomal membrane. Stabilization of lysosomal membrane limits the inflammatory response by preventing the release of lysosomal constituents of neutrophils such as proteases and bacterial enzymes. *In vitro* anti-inflammatory assay was performed on human red blood cell (HRBC) membrane because erythrocyte membrane is analogue to the lysosomal membrane. Collagen peptides in general expressed very low lysosomal membrane stabilization (Figure.4). However, there was a slight percentage protection on HRBC with the increase in collagen peptide concentration. The maximum membrane protection expressed were 4.01%, 3.66% and 3.27% by CP-5, CP-25 and CP-50, respectively at 0.2 mg/mL concentration. The standard, diclofenac drug, however, offered much higher membrane protection (83-91%). In an earlier study, the glandular extracts from albacore tuna (*Thunnus alalunga*) had expressed 28.86% protection at 400 mg/mL concentration Azeem *et al*. (2010).

Collagen peptides have exhibited little lysosomal membrane stabilization and thus showing no anti-inflammatory activity. The reason behind was further explored. There are two types of anti-inflammatory drugs-steroidal and non-steroidal. Peptides are non-steroidal drugs that elicit response by inhibiting prostaglandin; and the main function has been reported as COX inhibition by few authors (Harrington *et al*., 2008; Heo *et al*., 2010). COX-1 produces prostaglandin from arachidonic acid, while COX-2 produces inflammation-enhancing prostaglandin. Selective inhibition of COX-2 without influencing COX-1 by peptides prepared from salmon myofibrillar protein conjugated with oligosaccharides have been documented Saigusa *et al*. (2015). Peptides of myofibrillar proteins without sugar did not reduce inflammatory mediators like nitric oxide, TNF-α and IL-6. Hence, it is evidenced that peptides alone did not have anti-inflammatory action but if conjugated with sugar or oligosaccharide they elicit response.

In vitro Wound Healing Assay

Wound healing property was performed by *in vitro* scratch assay in 3T3-L1 cells in the presence of collagen peptides. The distance of cell migration was measured quantitatively for 48 h as it is directly proportional to the wound closure mechanism. The results showed that the maximum percentage of cell migration was recorded as 74.21%, 55.30% and 53.15% for CP-5, CP-25 and CP-50, respectively after 24 h treatment at 0.2 mg/mL concentration (Figure 5). The cell migration was rapid in CP-5 treatment than the other collagen peptides.

The 3T3-L1 is a cell line derived from mouse 3T3 cells, which is an adipocyte with fibroblast like morphology (Ruiz-Ojeda, Ruperez, Gomez-Llorente, Gil, & Aguilera, 2016). The fibroblast cell lines viz. 3T3-L1 and NiH-3T3 were earlier used for the assessment of wound closure by the plant derived compounds viz. glycyrrhizic acid and glabridin (Yip, Poh, & Chia, 2016). In an earlier study, tilapia skin collagen peptides expressed no obvious effect on the scratch closure at 50 µg/mL concentration after 6 h, whereas, significant wound closure was noticed after 12 h, 18 and 24 h treatment in HaCaT cells Hu *et al*. (2017).

Wound healing is a complex process involving four

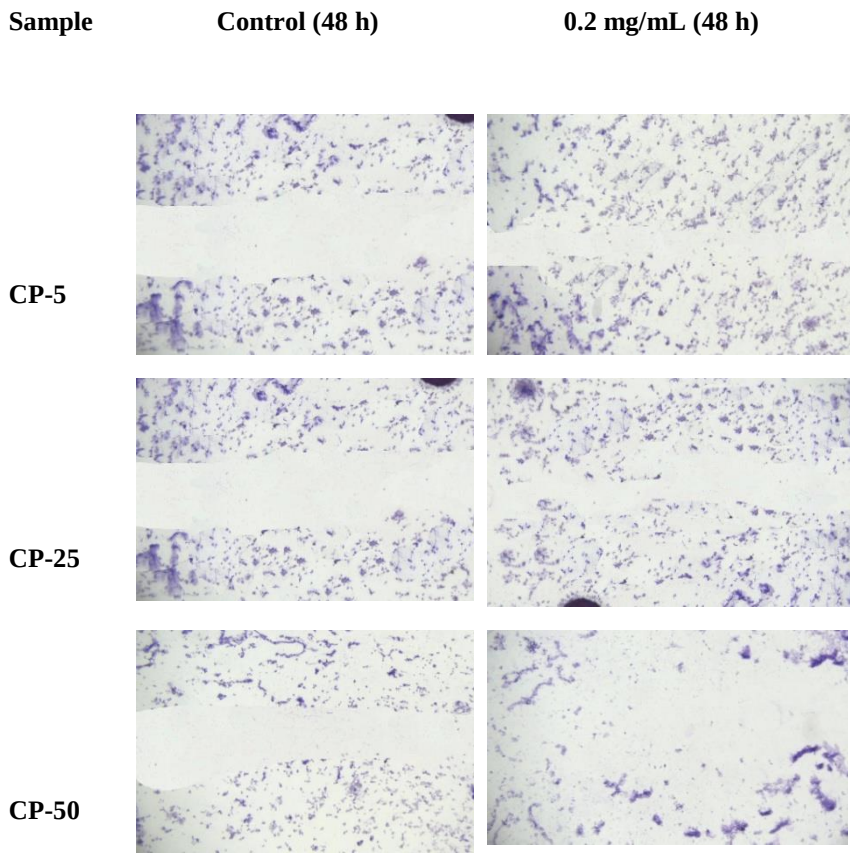

Figure 5. Measurement of 3T3-L1 cell migration in *in vitro* scratch assay.

phases; hemostasis, inflammation, inflammation, cell proliferation and migration; and tissue remodelling. The cell migration effect was in response to the down regulation of inflammation during wound healing Yip *et al*. (2016). The down regulation of chemokine ligand 5 (CXCL5) inflammation gene had activated the epidermal growth factor receptor (Dong, Kabir, Lee, & Son, 2013). Hence, it has been inferred that CP-5 had exhibited better cell migration than other peptides aiding faster wound regeneration.

Conclusion

The results indicated that hydrolysis of unicorn leatherjacket skin using collagenase at low temperature 5°C yielded collagen peptides having good *in vitro* anti-cancer, anti-diabetic and wound healing properties than those prepared at higher temperatures (25°C and 50°C). Fish skin collagen peptides in general did not express good anti inflammation activity. This study has enlighten the ways to utilize fish skin as substrate; and fish fins as enzyme sources for the preparation of collagen peptides with good bio-functional properties; which is an efficient method for bio-conversion of processing discards into valuable product, as well. More studies are further essential to prove each bioactive property in *in-vivo* animal models.

Acknowledgements

The authors thank to the Head of the Department of Fish Quality Assurance and Management, Tamil Nadu Fisheries University, Fisheries College and Research Institute, Tuticorin, Tamil Nadu and ICAR-Niche area of Excellence, New Delhi, India for his encouragement and for providing the necessary facilities to undertake this work.

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