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Use of Threadfin Bream derived collagen hydrolysate as shelf life enhancers.

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Abstract

Lipid rich food are known to undergo peroxyl linked breakage leading to the formation of free radicals that impart health problems upon consumption. This leads to decline in quality and shelf life hence reducing benefit to cost ratio. Current research hotpot India is the third largest fish producer in the world and generates over 0.3 million tonnes of waste during fish processing and packaging. This waste is rich in the extracellular protein collagen, which when fragmented displays many physiological activities. This study aims to utilize cryptic peptides from waste derived collagen hydrolysates and use them as functional food additives that inhibit lipid peroxidation and subsequently increase the shelf life of lipid based food. Collagen was isolated from Threadfin bream skin, swim bladder and scales by acid dissolution and salt precipitation. The purified collagen was analyzed through electrophoresis, spectral, elution pattern and was then subjected to enzymatic hydrolysis. CH presence was confirmed by running them through SDS-PAGE. A low molecular weight band was observed near the bottom of the gel indicating the presence of the hydrolysed collagen. CH were assayed for their lipid peroxidation inhibitory activity, hydrolysates obtained from SB and scales showed a 75-95% reduction in peroxidation while those obtained from skin did not show any inhibition activity. The shelf life of market available soya bean oil could be increased by 7.5%. CH was found to be non toxic when assayed for cell cytotoxicity as control and test plates displayed significantly similar cell adhesion and cell proliferation pattern. CH were also observed to be cytoprotective as they inhibited cell death upon addition of cumene hydroperoxide to cells.

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Keywords:

Collagen,
hydrolysate,
functional food,
lipid peroxide inhibition,
shelf life enhancers.

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1.Introduction

Foods can be regarded as functional if they can be satisfactorily demonstrated to contain bioactive ingredients that often go beyond the primary purpose of fostering normal growth and development. Lipid-based functional food are often subjected to peroxide induced deterioration leading to depletion in the quality of lipid content and nutritive values, short shelf life and increased pricing. Moreover, consumption of these potent toxic products can give rise to several diseases, including gastritis, atherosclerosis, nephritis and even cancer. This brings about the need for synthetic and natural antioxidants, which can prevent oxidative stress and its deleterious effects. Synthetic antioxidants such as butyl hydroxyl anisole are cost-effective and efficient but may display toxic and hazardous effects [1]. In the areas of human nutrition and biochemistry, natural antioxidants with no or little side effects have been the focus of growing interest for their potential health benefits.

Bioactive Peptides obtained from protein hydrolysates have been in demand for same time as LPO inhibition agents. However most bioactive peptides suffer from a common disadvantage; upon being ingested, they can evoke an immune response in the consumer if they possess sequences that resemble pathogenic epitopes [2].

Collagen, an extracellular triple helical architectural protein is highly conserved and immunocompatible, even amongst a wide range of evolutionarily distinct species and exhibits a high bioavailability owing to its unique sequence and structure [3]. Data gathered in the past decade confirm that collagen harbor specific fragments within their sequences, that when hydrolyzed, display physiological activity. Generally, these specific protein fragments are inactive within the sequence of the parent protein and when excised by enzymatic hydrolysis they can exert various physiological functions. Thus, collagen hydrolysate offers a better alternative than synthetic and/or other protein hydrolysates. If the hydrolysate can be obtained from a freely available source, its cost of synthesis will reduce and consequently the price of the food product will be economical.

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Collagen peptides can be easily extracted from the large amount of fish processing waste produced in India, a large portion of which is dumped as landfill [4, 5, and 6]. The waste can be recycled in the form of collagen hydrolysate which can be assayed for peroxide inhibitory and shelf life increasing activity. Fish collagen is dynamic in nature, easy to extract and free of most religious restrictions. This study therefore, aims at sustainable utilization of fish waste derived collagen to create a functional food additive that inhibits LPO formation and brings about enhancement of shelf life.

2. Materials and Methods

Butyl-hydroxy Anisole (BHA) was procured from SD Fine Chemicals, Bangalore, India. Linolenic acid, Sephadex G50 and molecular weight markers, including rat tail tendon collagen were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used for the experiments were of analytical grade.

2.1 Collection of fish waste and collagen extraction

The hybrid fish known as *Threadfin Bream* has been on the Indian market for quite some time and has high sales throughout the year, thus accounting for a major part of the fish waste. The skin, swim bladder and scales of *Threadfin Bream* was collected from fish market around Kumaraswamy Layout, Bangalore, Karnataka and collagen type I was isolated. The skin was descaled, dried, weighed and chopped into pieces. The samples were then suspended in 0.5M NaOH for the removal of non collagenous protein and stirred for 48h at 4°C, with change in NaOH solution every 6h. The samples were then dried, weighed and defatted by suspension in 10% propanol. This was followed by suspension in 0.5M acetic acid and precipitation with 6% NaCl solution. The precipitated collagen subjected to dialysis. The samples obtained were stored in sealed bottle at 4°C.

2.2 Characterization of purified collagen

The isolated collagen was characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) band pattern [7] with a 7% resolving gel. Collagen samples were mixed with gel loading buffer, incubated at 75°C for 1.5min, cooled to room temperature and loaded in the well. Type I collagen purified from rat tail tendon treated in the same manner was ran as positive control. Gels were run at 50mV for 3h. On completion, gels were stained for 45 min with Coomassie Brilliant Blue and destained according to standard protocols.

Briefly, the column was cleaned with water and then saturated with 0.1M acetate buffer. 100µl of the sample was made up to 500µl with acetate buffer and loaded into the column. The buffer was run at a constant flow rate of 1 ml min^{-1} and the volumes at which peaks appear were noted. After the run, the column was purged with deionized water and stored in 20% ethanol.

2.3 Preparation of the Hydrolysate

A 30mg ml^{-1} suspension of collagen was prepared in 0.25 M phosphate buffer, pH 7.5 and incubated at 37ºC for 30 min. Collagenase was added to the buffered collagen at a concentration ratio of 1:300 and the mixture incubated at 37ºC for 12 h. After the incubation time, the reaction was arrested by addition of 0.1 M acetic acid. The mixture was centrifuged to remove unhydrolyzed collagen debris.

2.4 Characterization of the hydrolysate

Presence of the hydrolysate was confirmed with gel-permeation chromatography using an ÄKTAprime plus FPLC unit. 2ml of extracted hydrolysate was dissolved in 0.05M acetate elution buffer and applied to a sephadex G100 column equilibrated in the same buffer. Elution was carried out with a flow rate of 1ml min^{-1} for 50 min until all the hydrolysate eluted out. As control, collagen was run in the same column for an equal amount of time. Absorbance was monitored at 280nm and 2ml fractions were collected.

2.5 Lipid peroxidation inhibition assay

The methodology given Li et al [8] was followed to measure the extent of lipid peroxidation. 1.5 ml of 50 mM linolenic acid in absolute ethanol was mixed with an equal volume of phosphate buffer, pH 7 and 2 ml of the hydrolysate in phosphate buffer was added. BHA was used as the positive control. The final solution was incubated in the dark at 60ºC in a screw-cap glass tube. Triplicate glass tubes were used for each sample. The degree of oxidation was measured at 24 h intervals using ferric thiocyanate for colour development. 100 µl of the reaction mixture was isolated every 24h and mixed with the following reagents: 4.5 ml of 75 % ethanol, 100µl of 30 % ammonium thiocyanate, 200µl of 1 N HCl and 100µl of 20 mM ferrous chloride solution in 3.5 % HCl. The colour developed was measured at 500 nm.

$$\%\ of\ reduction = 100 - \left(\frac{100 \times slope_{Test}}{slope_{Control}}\right)$$

2.6 Peroxidation inhibition assay in Vero cells

Vero cell lines were maintained according to the standard protocol [9].
The methodology of Gavino et al as modified by Das et al [10] was used to study LPO inhibitory properties of CH in Vero cells. 1.5×10^{4} cells were seeded onto the 35mm dishes. Cell count was checked at intervals till cells were 80% confluent at a count of $3.5\pm0.7\times10^{6}$ cells per dish. A fixed volume of the medium was aspirated out and replaced with medium containing the hydrolysates at a final concentration of 1mg/ml. Stock fatty acids stored at 4°C was serially diluted in sterilized Dulbecco's modified Ca^{2+} and Mg^{2+} free phosphate buffered saline and added to the cells such that the final concentration would be 120µM. Cumene hydroperoxide (CHP) was added to the dishes at final concentrations ranging from 0-100µM. The control group comprised of a similar number of cells seeded on dishes without any addition of fatty acids or LPO generator. After 6h incubation, adherent cells were counted and the stressor concentration ensuing 50% cell survival was calculated.

2.7 Increase in shelf life

CH were also assayed for their ability to increase the shelf life of lipid based food. In this assay CH were mixed with Soya bean oil and stored for a period of 45 days. After every nine days, aliquots were collected and LPO levels were assayed based on the thiocyanate method. BHA was used as the positive control to test the CH efficiency to inhibit oxidation over a period of time and intact collagen was used as the negative control.

Fig 1 shows the results obtained when CH were assayed for their ability to increase shelf life of lipid based food. As seen, peroxidation levels in samples containing CH are maintained at a low level, with almost the same efficiency as that of BHA. It can also be observed that intact collagen does not show this activity. The shelf life of market available soya bean oil could be increased by 7.5% due to the use of collagen hydrolysates.

2.6 Statistical analysis:
The LPO inhibition assay was carried out in triplicate. The graph was drawn with the mean of three absorbance values. The dataset was analyzed via two-way ANOVA and individual data sets were analyzed via post hoc Tukey's test. Probability values (p) greater than 0.5 were considered to be insignificant.

Samples	Weight 1 (g)		Weight 2 (g)		Weight 3 (g)		Weight 4 (g)		Weight 5 (g)	Soulable collagen	Sol Coll/Vol (g/ml)
Skin	22.02	**NaOH**	18.07	**Butanol**	16.07	**EDTA**	16.07	**Acetic Acid**	12.01	7.95	0.1
Scales	24		23.1		22.9		19.05		5.95	7.15	0.047
Swim Bladder	3.8		0.2		0		0		0	3.6	0.01

Table1: Purification of collagen from Threadfin Bream

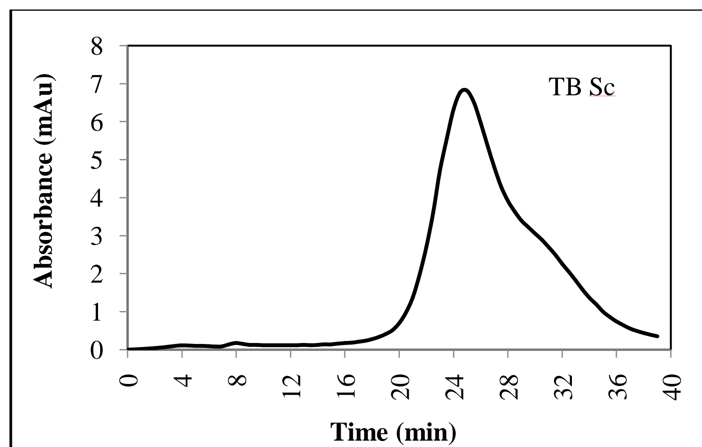

Fig 2a: Elution profile of Intact Collagen samples obtained from TB scales run in a 10 cm Sephadex matrix . One peak eluding at 25 minutes can be observed.

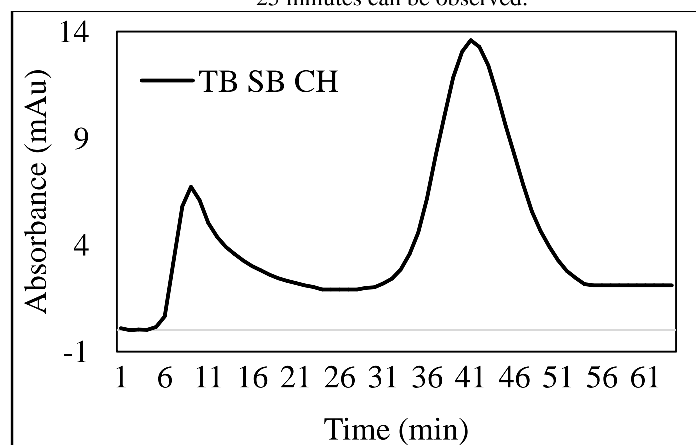

Fig 2b: Elution profile of CH obtained from TB SB run in a 10 cm Sephadex matrix at a constant flow rate of 1ml/min. Two peaks one at 11 minutes and the other obtained at the 43 minute can be observed.

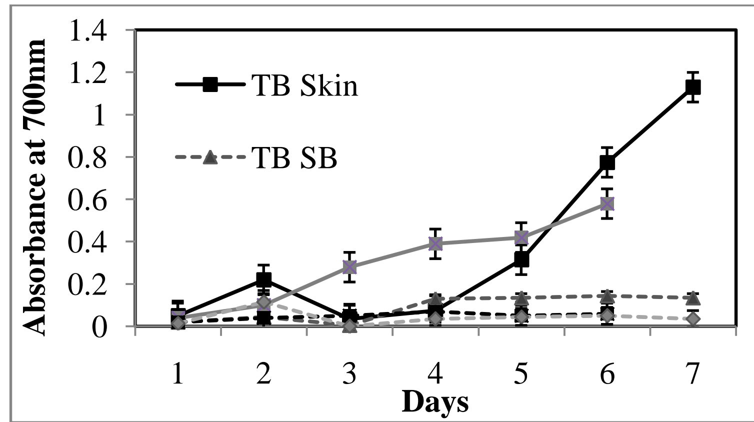

Fig 3: Lipid Peroxidation Inhibition Assay done for TB SB, Skin and scales with LA as the negative control and BHA+LA as the positive control.

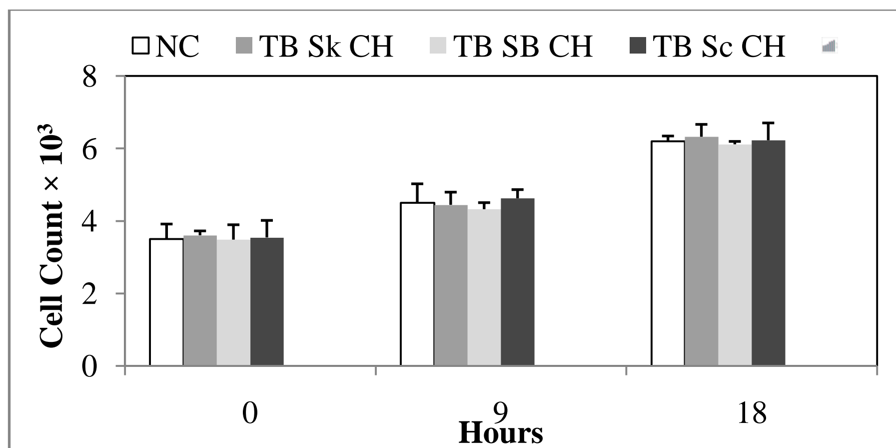

Fig4a: Cell adhesion and proliferation assay done to check for toxicity of CH obtained from TB Skin, SB and Scales

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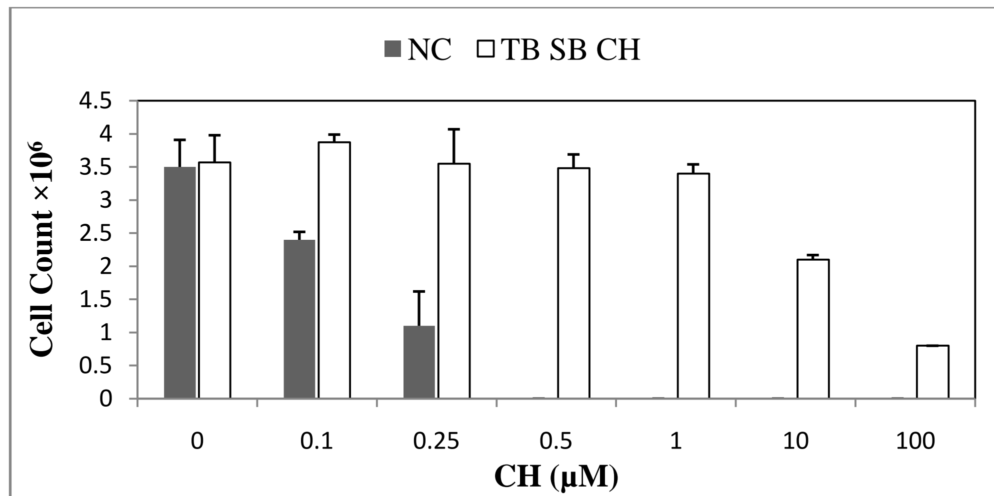

Fig 4b: Cytoprotective activity of CH obtained from TB SB when assayed against cumene hydroperoxide.

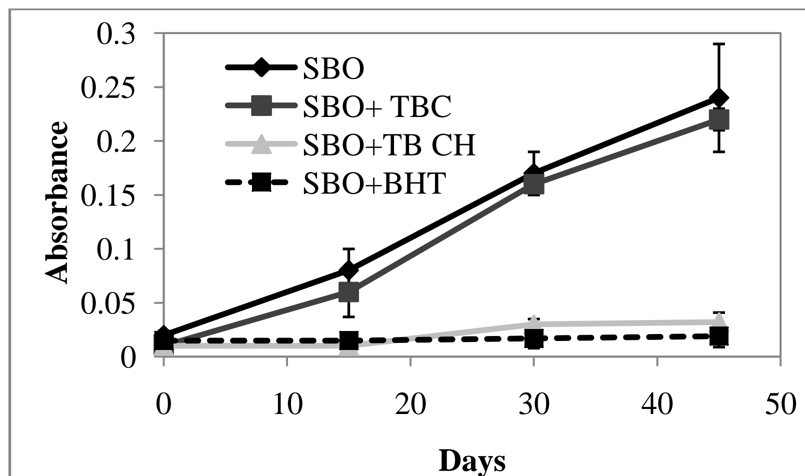

Fig 5: Increase in Shelf life upon usage of collagen hyddrolysate mixed with market available soya bean oil

3. Results:

3.1 Extraction and purification of collagen obtained from Threadfin Bream

Table 1 gives the % extraction of acid soluble collagen. The skin and scales constituted of 18% and 3.8% of non-collagenous protein respectively, which was removed by NaOH treatment.10.9% and 1.2% of fat soluble compounds were removed by butanol. The remaining material was extracted with acetic acid. Skin yielded 36.1% of soluble collagen while scales yielded 9.16%. 100% soluble collagen was obtained from the swim bladder. The % occurrence of the unique imino acid hydroxyproline, a constituent of collagen involved in the stabilization of the triple helix, is customarily used to calculate

the purity of isolated collagen. Collagen displays characteristic absorbance or λ_{max} of collagen, which peaks at 220nm. The 280nm peak is significantly diminished in collagen due to the relatively low abundance of aromatic amino acid residues tyrosine and tryptophan. The Threadfin derived collagen samples displays its specific band pattern with two α bands and high molecular weight β and γ bands. The bands obtained resembled that of rat tail collagen, a pure source of collagen type I, which is used as positive control in collagen identification. The increased in density of the α1 band compared to that of the α2 band is due to the arrangement of chain of collagen Type I. Collagen comprises three α chains among which two polypeptides are of α1 and the remaining one, α2 ([α1(I)]2[α2(I)]). This gives a double band in electrophoretic gels, with one having double the intensity of the other, a characteristic of collagen. The gel permeation elution profile displayed peaks at the 14.5 min, 20.5 min and the 24.5 min respectively for SB, Scales and skin. The run time was 45 minutes for all the samples. (Only one figure shown)

3.2 Collagen Hydrolysate Characterization

Enzymatic digestion of parental proteins is an effective method for obtaining small peptides. Hydrolysis also results in the production of higher number of free N-terminal and C-terminal amino acid residues, which in turn, affects its availability in physiological systems. In order to release the maximum number of peptides as possible, collagen has been subjected to heat treatment followed by protease V8 cleavage and the hydrolysate extracted through acetic acid treatment. The presence of low molecular weight peptides in the hydrolysate was confirmed by gel permeation chromatography and SDS-PAGE. Fig. 2a displays the FPLC elution pattern of collagen, which elutes out from 6-10 min due to its high molecular weight. Fig. 2b gives the elution pattern of hydrolysate which comes out after 20ml of elution, indicating its lower molecular weight. A short shoulder peak indicates a small amount of recoiled hydrolysate which elutes out at 6-10ml.

3.3 Lipid peroxide inhibitory activity of collagen hydrolysate

Lipid peroxidation proceeds through a free radical mechanism that is catalyzed by heat and the presence of oxygen. The amount of peroxide produced is quantified by reaction with Fe^{2+}, which is converted to Fe^{3+} and reacts with ^{-}SCN to give a blood red coloration. The results in Fig 3 makes it clear that the FCH, FC and BHA displayed significantly ($p<0.05$, by ANOVA) different levels of LPO inhibition. There is a significant difference between the LPO reduction by FCH/LA and LA ($p<0.001$) and between FC/LA ($p<0.001$), indicating that the hydrolysate distinctly exhibited the properties of LPO reduction while collagen didn't. By far, BHA was the most effective ($p<0.01$) in inhibiting LPO formation. The results obtained make it absolutely clear that collagen hydrolysate was quite effective in blocking LPO formation (Fig 3)

3.4 Mammalian cell culture assay

The control and the test plates displayed significantly similar ($p>0.05$) cell adhesion and cell proliferation pattern (Fig. 4a), indicating the CH did not hinder growth of mammalian cells and were labelled as non toxic.

Cumene hydroperoxide is known to generate LPO in cell culture in the presence of C18 lipids and had a significant effect on the cell viability.[11]. The hydrolysate was also able to reduce cell death occurring due to cumene related LPO. The negative

control shows complete cell destruction after 0.25µM CHP. In the presence of the collagen hydrolysates, even at 1µM CHP cells were found to be viable and declines beyond this value. (Fig 4b). Plates without hydrolysates display a drastic decrease in live cell as the stressor concentration is increased.

4. Discussion:

Antioxidative properties of the peptides are more related to their composition, structure, and hydrophobicity. The exact mechanism underlying the antioxidant activity of peptides has not fully been understood, yet the bioactive nature of protein hydrolysate is undeniable, based on the number of reports. Tyr, Trp, Met, Lys, Cys, and His are examples of amino acids that cause antioxidant Activity. Collagen, however, generally lacks the above residues and is mainly comprised of a GXY triplet where X is often proline and Y is often hydroxyproline. The amino acid sequence in collagen comprises of a larger content of hydrophobic residues and a smaller content of aromatic amino acids and cysteine, which is generally required for a peptide to be antioxidative. And yet, collagen hydrolysate was able to display LPO inhibitory activity in this study. For any compound to display LPO inhibition, it has to be soluble in lipid and in this respect, it can be safely concluded that the hydrophobic residues in collagen are responsible for the lipid solubility. Research carried out on collagen peptides in the last 5-6 years have unearthed certain sequences such as PG and QGAR, both common to type I collagen, which can imbibe collagen hydrolysate with antioxidative properties [12,13].

Collagen yield obtained from the skin of Threadfin Bream was found to be 36.1% which is significantly less than the yield of 78.86% obtained from the skin of Pacu [11] but higher than10.7%, the yield obtained from Ocellate puffer fish [14]. The scales of threadfin bream yielded 9.16% of collagen which is much lower than the yield of 50.9% obtained from sardines[15]. The yield efficiency from the swim bladder was found to be 100%, as SB are known to be composed of primarily of collagen. This variation in the yield of collagen is due to minor variations in protocol and also due to the variation in tissue structure of different fishes.

The fact that hydrolysis can increase the functional properties of food has been established previously. Ingestion of fermented soybean extracts lead to increase in redox enzyme activities in liver and kidney [16] and reduce paraquat-induced oxidative stress in male Wistar rats [17]. In addition, intake of egg white hydrolysates (0.5 g/kg/day of egg white hydrolysates) increased the radical-scavenging capacity of plasma and decreased lipid peroxidation products in the aorta for spontaneously hypertensive rats [18]. CH hydrolysates were also found to decrease the production of cumene hydroperoxide in the assayed vero cells hence displaying cytoprotective activity as well. This further cements that CH act as efficient peroxide inhibitors. Shelf life is said to be the period of time during which any material may be stored and remains suitable for use. Lipid peroxidation has been know to undergo peroxidation hence leading to them not being able to be stored for long due to risk of spoilage. A recent study claimed that as much as 40% of food is lost in their processing from crop to processed food [19]. Lipids play a multifunctional role in flavour, as they influence taste both on a physical and chemical scale, as a reduction in fat results in a higher flavour loss due to flavour volatility [20].

In a world where demand for food is at a constant increase, loss of food due to decreased shelf life is one of the areas which have not been vastly studied. Proteins have been previously used for the increase in shelf life of food in the form of protein film. Protein-based edible films can be applied inside heterogeneous foods at the interfaces between different layers of

components. They can be tailored to prevent the deterioration of inter-component moisture and solute migration in foods [21]. Viable edible films and coatings have been successfully produced from whey proteins [22], peanut protein [23] and from milk protein.[24]. CH however provide a much more cost effective and easy method for the both the inhibition of peroxidation and the problem of decreased shelf life in lipid based food. Due to their non toxic nature, the hydrolysates are safe and can be used in day to day life. Previous research has also showed that the inclusion of edible/biodegradeable films may impact several properties of the system and hence affect consumer acceptability.

The results of this study show that the lipid peroxidation inhibition activities of the fish collagen hydrolysate samples are significantly different from each other ($p>0.05$). Hydrolysates obtained from SB and scales showed a 75-95% reduction in peroxidation while those obtained from skin did not show any inhibition activity.

The hydrolysates were proven to be noncytotoxic and were able to inhibit H_2O_2 induced free radical generation in mammalian cell culture, indicating that they were effective as consumable peroxide inhibitors.

Conclusion:

Collagen is a structural protein and is a primary component of fish skin, scale and bones. When hydrolysed this protein shows various activities that are unrelated to that of the intact protein. Such peptides are termed as cryptic peptides and show a wide variety of functions of which lipid peroxidation inhibition is one. The realm of cryptic peptide has recently been uncovered and studies such as these confirm the potential of structural protein hydrolysates as a source of cryptic peptides. Hydrolysates derived from collagen show LPO inhibition and hence can be used to increase the shelf life of lipid based food.

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