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Biofunctional food additives – emulsifiers and humectants isolated from *Catla* skin and bladder collagen

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Abstract

A staggering 1.3 billion ton of edible foods are wasted globally per year, primarily due to loss of texture and moisture during storage. Current emulsifiers/humectants like PEG and sorbitol used to stabilize food texture and composition are known to display toxic effects at higher doses. This has lead to a search for naturally available and non toxic alternatives. Since hydrolysates of the extra cellular protein collagen are known to display several physiological activities, this study was conducted to evaluate the potential of collagen hydrolysates (CH) used as emulsifiers/humectants in food. Collagen was isolated from the skin and swim bladder of *Catla catla* and was identified to be collagen type I based on its electrophoretic and elution profile which displayed double peaks at 100 kDa. Bacterial collagenase was used for hydrolysing and fragmentation was confirmed by altered electrophoretic and elution profile displaying mass of 7-12kD. CH exhibited an emulsification activity of 2.5±0.78 m^2/g at 0.005% concentration and emulsion stability of 33±1.2 minutes, significantly similar [$p<0.05$] to the positive control. The CH sample could successfully retain 97±0.25 % of added water during a period of seven days. The CH sample displayed a mere 2+1.04% haemolytic activity and was confirmed non-toxic. CH can revolutionize the current food and health industry as they display high bioavailability and bio compatibility with no side effects, and can be isolated in bulk from marine industry processing waste.

Keywords:

Collagen hydrolysate
Food additive
Emulsifier
Humectant
Non-toxic.

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1. Introduction:

Food storage has become a prime feature in the present decade for maintaining the physical and physiological integrity of food products. Food loss refers to decrease in edible food mass which was originally meant for human consumption [16]. According to the Global Food Losses and Food Waste [2011] reports, there is a loss of 1.3 billion tons of food due to depletion in moisture and texture during storage.

Humectants and emulsifiers are the most common food additives which are utilized to aid the loss of food, arising due to storage problems. Humectants help in moisture retention while emulsifiers allow uniformity between diverse phases in consumables leading to increase in stability and shelf life. Some of the commonly used synthetic food additives are Polyethylene glycol and sorbitol. However, despite decreasing maintenance charges and product cost, they pose various health hazards like kidney problems, skin irritation and can also produce toxic compounds upon degradation [1], [9], inducing the current research hotspot to incline towards natural food additives.

Peptides are a class of bio-molecules that are now being preferred over synthetic additives for their diverse range of bioactivity and lower production cost [10], [11]. The drawbacks of using these peptides include their low availability and ability to provoke an immune response. The modern scenario focuses on searching for non-toxic and non-immunogenic peptides with high bioavailability and desired properties.

Collagen protein hydrolysates satisfy the above criteria and is abundantly available in protein rich fish waste [2]. Annually 0.3 million tonne of waste is generated in India during fish processing and packaging. Apart from utilizing this waste in production of glue and isinglass, majority of it is dumped as landfills which lead to increase in pollution and foul odour [15].The study aims in production of biofunctional food additives from collagen hydrolysate isolated from *Catla* skin and swim bladder waste.

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2. Materials and Methods:

Carboxy methyl sepharose, Sephadex G100 and molecular weight markers, including rat tail tendon collagen were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

2.1 Collection of fish waste and collagen extraction:

The skin and swim bladder of *Catla catla*, was collected from nearby fish market. Skin was descaled and samples were subjected to successive treatment with 0.5M NaOH, decalcifying medium (DCM) and 10% butanol for removal of non-collagenous components. Dry weight of samples were taken at every intermediate steps. The sample was now treated with 0.5M acetic acid to obtain soluble collagen. The filtered collagen was homogenized and precipitated with 6% NaCl solution, dissolved in acetic acid and subjected to dialysis. The samples were lyophilized and stored at 4^0C.

2.2 Characterization of purified collagen:

The isolated collagen was loaded onto dialysis tubing with cut off 6.04kDa and dialyzed against water for 6h to remove acetic acid. The dialyzed collagen was characterized by chromatographic techniques and SDS-PAGE band pattern. The purity of the extracted collagen was assessed by Laemmli's SDS-PAGE method (1970) with a 7% resolving gel. Collagen samples were mixed

with gel loading buffer, incubated at 100°C for 1min and loaded in to the well. A standard molecular marker was used for comparison of bands. Gels were run at 50mV for 3h. On completion of run, gel was stained for 45min with Coomassie Brilliant Blue and destained according to standard protocols.

An AKTA Prime FPLC unit was used for gel permeation (GPC) and ion exchange (IEC) chromatography for further characterization. A 10cm sephadex G100 column was set up for GPC. 0.5ml of collagen sample was made up to 1ml with acetate buffer, pH 4.5 and the solution was incubated at 45^0 C for 10min. The column was saturated by passing 0.1M acetate buffer. The dead time and equilibrium time were noted down and kept constant for all the samples. The sample was loaded into the column at the flow rate of 1.0ml/min and the elution buffer was run for 30min. Post run the column was washed with deionized water and flushed with 20% ethanol before dismantling.

A 4.5cm CM sepharose column was used for IEC. The matrix was saturated by running 0.5M acetate buffer, pH 4.5 after which the sample was injected. Binding buffer was allowed to flow at 1.2 ml min^{-1} following which salt concentration was varied from 0-100% for a period of 25 min. The elution time and volume of the peaks were measured. Rat tail tendon collagen was run as standard marker. The column was washed and stored in 20% ethanol solution until dismantled.

2.3 Hydrolysate characterization:

Lyophilized collagen was suspended in 0.5M phosphate buffer at a concentration of 300 mg ml^{-1}. 2 ml of the enzyme was added to the buffered collagen under sterile conditions and the mixture incubated at 37ºC for 24 h. As controls, vials containing only collagen and only enzyme were used. After the incubation time, the reaction was arrested by addition of 0.1 M acetic acid. The undissolved debris was discarded after centrifugation at 7000rpm in a Remi C24 centrifuge. The supernatant was collected, frozen at -80°C and lyophilized in a Thermo freeze drier. A fixed quantity of the dried CH was subjected to hydroxyproline estimation by Woessner's method to know the quantity of hydroxyproline. The solid CH was also subjected to a 10% SDS-PAGE to determine the elution pattern. The molecular weight of the CH was determined by running the

samples in a calibrated sephadex G100 column. 0.5ml of hydrolysate was made upto1ml in 0.05Macetate 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. Absorbance was monitored at 280nm.Void volume was calculated by running 2mg Blue Dextran.

2.4 Emulsification Assay:

The protocol of Pearce and Kinsella [1978] was followed to check the CH for their emulsifying property in lipid based foods. Appropriate amount of CH were dissolved in 0.25M phosphate buffer to obtain a final concentration of 0.005%. 0.38ml soybean oil was mixed with the diluted collagen samples and 20ml of deionized water was added to make up the volume for vortexing. The mixture was vortexed at 15000rpm for 1min. The absorbance of the vortexed samples were taken at 520nm with time durations from t=0 to t=10min, after emulsion formation. The emulsifying activity index (EAI) and the emulsion stability index (ESI) was calculated as follows:

$$EAI\left(\frac{m^2}{g}\right) = \frac{2.303 \times Dilution\ factor(200) \times A}{c \times \theta \times 10000 \times p}$$

Where, A is the absorbance at 520 nm; c is the protein concentration (g/ml); Ɵ is the disperse phase volume fraction (0.25); and p is the optical path (0.01m).

$$ESI(\text{min}) = \frac{A_0 \times t}{A_0 - A_{10}}$$

Where, A_0 is the absorbance at 520nm at time 0min; A_{10} is the absorbance at 520nm at time 10min; and t is time.

2.5 Water Retention Assay:

The CH samples were assayed for their water retention property in accordance to Hou et al. method. 0.5g of lyophilized CH samples were mixed with 1g water and the weight was noted. The time was taken as t=0. The mixture was sealed in a dessicator and the sample weight was taken in intervals of 24hr. Intact collagen was taken to be the negative control and glucose was used as positive control. The entire protocol was performed in duplicates and the % of water retained was calculated by the following formula:-

$$\%\ \text{of Water Retained by the sample} = 100 - \left[\frac{(Slope_{test}) - (Slope_{control})}{[Slope_{control}]}\right]$$

2.6 Hemolytic Assay:

Hemolytic assay was done to check for the toxicity of the collagen samples obtained. 5 ml of blood was diluted 10 times with phosphate buffer saline (PBS). From this 1ml of blood was taken in a test tube and made upto 10ml with PBS followed by addition of 500µl of neutral collagen hydrolysate sample. The absorbance of the samples was taken at 540nm using a colorimeter. Absorbance was measured at 0^{th} and 30^{th} minute. For the positive control, tween 20 was mixed with blood. 1% glycine solution was used as the negative control.

$$\%\ hemolysis = \left[\frac{Abs_{Test} - Abs_{NC}}{Abs_{PC}}\right] \times 100$$

Where Abs_{test}, Abs_{NC} and Abs_{PC} equals the difference in absorbance at 0^{th} min and 30^{th} min for the following samples: CH, negative control and positive control respectively.

2.7 Statistical analysis:

The emulsification assay was carried out in duplicate. The graph was drawn with the mean of two 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.

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Figures and Tables:

	Dry weight (g)	NaOH	Weight 1 (g)	DCM	Weight 2 (g)	Butanol	Weight 3 (g)	Acetic Acid	Insoluble (g)	Collagen (g)	% collagen (wt/g of material)
CaSk	111.34		89.3		87.4		74.1		3.2	70.9	63.67
CaSB	4.2		3.9		3.9		3.88		0	3.88	92.38

Table 1 - % Extraction of soluble collagen by *Catla* Skin and Swim Bladder.

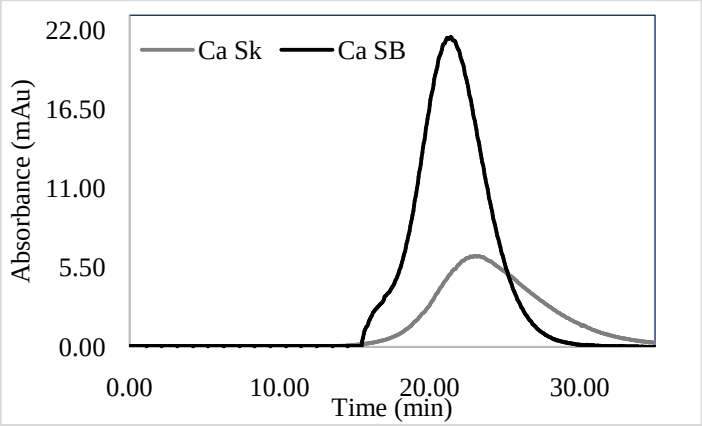

Fig. 1 - Gel Permeation elution profile of Catla Skin and bladder collagen. The peak was obtained at 23 minutes of the 35 minutes run time.

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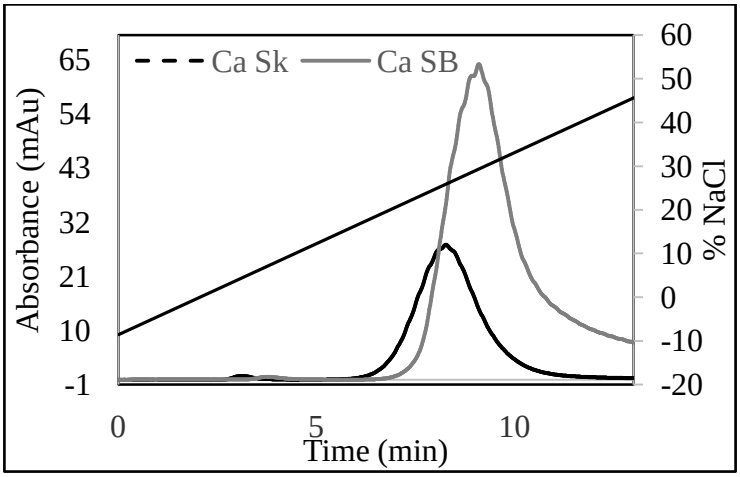

Fig. 2 - Ion Exchange elution profile of Catla Skin and bladder collagen. Peak 1 was obtained at 8±1.5% salt concentration and peak 2 was obtained at 28±1.2% salt concentration.

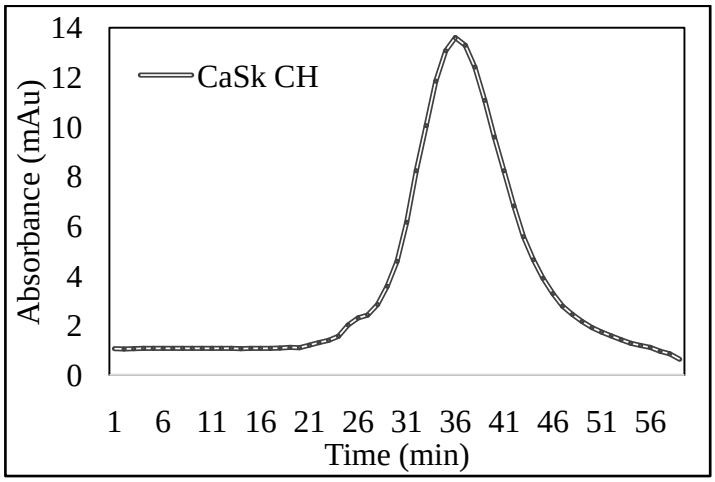

Fig. 3 - Gel permeation elution profile of Catla skin collagen hydrolysate. Peak was obtained at 36 minutes.

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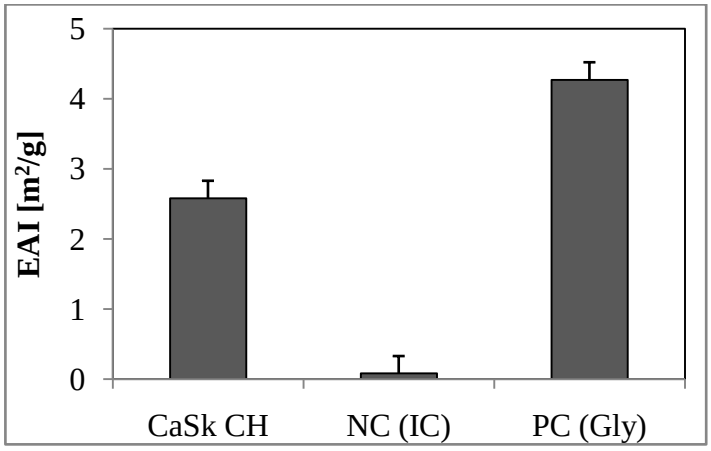

Fig. 4 - Emulsification Activity Index comparison of Catla skin collagen hydrolysate.

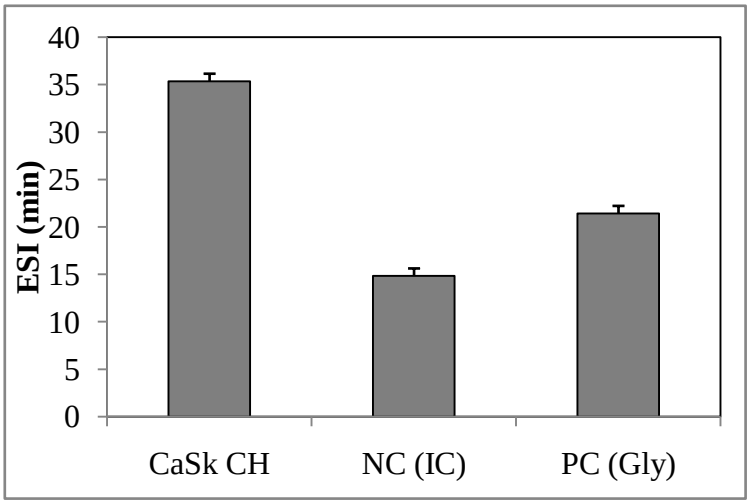

Fig. 5 - Emulsification Stability Index comparison of Catla skin collagen hydrolysate.

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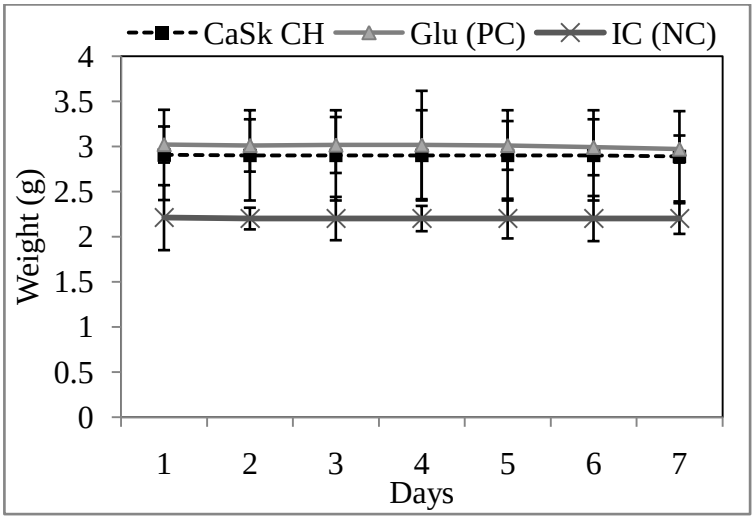

Fig. 6 - Water Retention Activity comparison of Catla skin collagen hydrolysate.

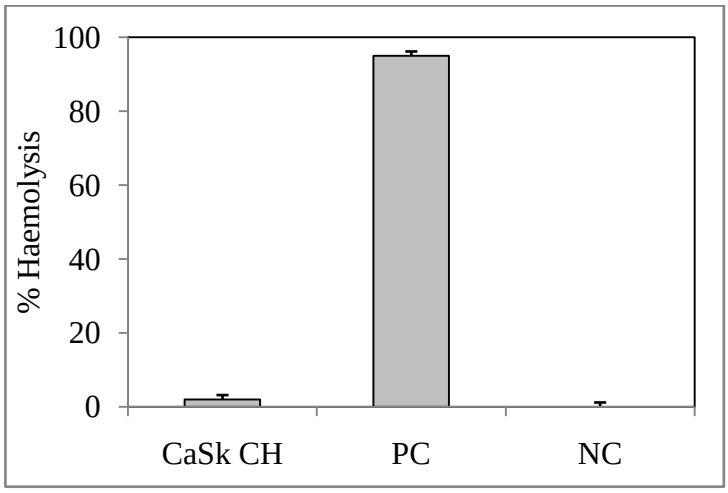

Fig. 7 - Evaluation of toxicity of skin collagen hydrolysate.

3. Results:

3.1 Extraction of Catla skin and bladder collagen:

The % extraction of acid soluble collagen is depicted in table 1. The skin constituted of 19.77% of non-collagenous protein, which was removed by NaOH treatment, 1.7% metal ions removed by DCM and 11.96% of fat soluble compounds, removed by butanol. The remaining material was extracted with acetic acid and acid soluble collagen was found to constitute 63.67% of the raw material. On the other hand, swim bladder comprised of 7.16% of non collagenous protein, 0.46% of fat and 92.38% of acid soluble collagen.

3.2 Characterization of collagen:

3.2.1 Electrophoretic profile of collagen:

A 7% gel was used for sample run. It was observed that skin and swim-bladder exhibited distinct formation of α bands and higher order bands, which are unique to only type I collagen. The band pattern was found similar to that of rat tail tendon which was used as a marker for collagen type I. The triple helical arrangement of collagen type I is composed of two α1 and one α2 polypeptide chains. The presence of α1 polypeptide in double the amount of the α2 chain gives rise to the increased density of α1 band when compared to α2 band.

3.2.2 Elution profile of collagen:

The gel permeation elution profile of the collagen samples displayed a maximum absorbance at 23min as displayed in Fig. 1. The run time was 35 min for the samples. As seen, the samples exhibited one peak which is due to the uncoiled individual α-polypeptide chains. Substituting the V_t/V_o value in the graph, the molecular weight corresponding to the second peak was calculated to be 116kDa.

As displayed in Fig. 2, IEC resulted in a minor peak at 8±1.5% salt concentration, possibly due to higher order structures and a major peak at about 28±1.2% salt concentration indicating presence of a moderately positively charged molecule. The values were significantly close to the positive standard, rat tail tendon collagen type I collagen which was run in similar conditions and eluted out at 30% salt concentration (Not shown in figure).

3.3 Characterization of hydrolysate:

The hydroxyproline count of the CH came as follows; bladder CH 8.4±0.8mg/g, skin 5.9±0.8mg/g and scales 5.1±0.2mg/g. It was confirmed that collagen has indeed been hydrolysed by running the hydrolysate in a 10% SDS-PAGE gel. The band pattern ranging near 6kDa confirms the fragmentation of collagen. Further characterization was confirmed with gel-permeation chromatography using an ÄKTAprime plus FPLC unit. A single peak as shown in Fig. 3 corresponding to hydrolysate was obtained at 36min. The peak corresponded to a molecular weight of 9.8kDa. However, the CH could still have smaller peptides. Collagen peptides tend to recoil with each other and form larger aggregates.

3.4 Activity of collagen hydrolysate:

EAI and ESI values of *Catla* Skin collagen hydrolysate at concentration of 0.005% is shown in Fig. 4 and 5. EAI defines the amount of surface area stabilized by the CH and ESI denotes the time period for which the emulsion was

stable. It was observed that EAI value of skin collagen hydrolysate was 2.5±0.78 m^2/g which was lesser than glycine (positive control). However, the sample displayed higher emulsion stability (33±1.2 min) than positive control.

The hydration ability of protein to hold its own and added water under influence of external forces is termed as its water holding capacity (WHC). The WHC of skin collagen hydrolysate is shown in Fig. 6. It can be inferred that CH sample could successfully retain 97±0.25 % of added water during a period of seven days. The ability of CH to retain water is equivalent to that of glucose which was used as a positive control.

3.5 Toxicity of CH:

The hydrolysates were evaluated for their toxicity via haemolytic assay. As displayed in Fig. 7. SkinCH samples displayed a 2+1.04% haemolytic activity which is similar to the negative control used. This shows that collagen hydrolysates are non toxic to the red blood cells confirming their biocompatibility.

4. Discussion:

Annually, India generates more than 0.25million tonnes of fish waste which is produced while food processing and production [3]. A part of this waste is utilized for production of isinglass, amino acids and other essential components, but majority of it is dumped as landfills which lead to leaching of chemicals and development of foul odour. This discarded waste is rich in an extracellular protein collagen, peptides of which has been proven to exhibit various bioactive properties like lipid peroxidation, wound healing and various other food enhancement abilities. This triple helical protein is composed mainly of G-X-Y amino acids, where majority of times X is proline and Y is hydroxyproline [13].

Table 1 describes about the extraction efficacy of soluble collagen. As seen from the data, *Catla* skin (63.67%) and swim bladder (92.38%) resulted in higher yield than that of Rohu and deep-sea red fish skin which was found to be 18.8% [4] and 47.5% [14] respectively.

A number of proteins have been reported to exhibit emulsifying properties due to the presence of both hydrophobic and hydrophilic groups [7]. The capability of protein to help in the formation of an emulsion is estimated by EAI, while the time being for which the emulsion remains stable is gauged by ESI [12]. The study confirms that skin CH exhibited 2.5 m^2/g EAI value at 0.005% which was found to be better than study done by Giménez et al. [2008] on squid and sole skin which yielded in 25 m^2/g EAI value at 1% concentration. The hydrolysates were able to retain 97% of water over 7 days. This could be attributed to the high abundance of Hyp residues in collagen which can interact with water molecules through hydrogen bonds and consequently, retain moisture.

Conclusion:

To summarize, with substantial increase in the demand for lipid-based processed delicacies, there is also an equal rise in awareness for improvisation of health through intake of functional food additives to enhance the overall robustness. Functional food additives are compounds required for regular food up-gradation in terms of nutrition, texture, taste and shelf life. With 20 billion dollar sales per annum, they have became an integral part of food industry and is a growing industry. However, because these foods contain sensitive components, food spoilage due to storage conditions is a common problem. On the other hand, meat and fish industry produces a large amount of collagen-rich waste, which if recycled will not only add value to the waste but decrease pollution. Utilization of these protein rich wastes as food stabilizing agents will open a novel doorways both in waste management and health food industry.

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