Research Article

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Recycling fish skin for utilization in food industry as an effective emulsifier and foam stabilizing agent

Etkili emülgatör ve köpük stabilize edici ajan olarak gıda endüstrisinde kullanım için balık derisinin geri dönüşümü

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Abstract

Background: Indian fish industry produces a staggering amount of waste every year part of which is dumped as landfill leading to pollution.

Objective: This study aims to extract collagen from fish skin, a major component of this waste, hydrolyze it and use the hydrolysate as emulsifiers to increase the shelf life of food products.

Materials and methods: Collagen was purified from the skin of Catfish, Mullet and Indian Salmon were

characterized through infrared and diffraction studies. The samples were hydrolyzed enzymatically. The hydrolysates were assayed for emulsifying, foam stabilizing and hemolytic properties.

Results: The samples displayed an electrophoretic and infrared spectral profile corresponding to that of collagen type I. X-ray diffraction patterns revealed the presence of triple helix and traces of phosphate. The hydrolysates (6 kDa) exhibited the highest emulsion at 2.74 ± 0.20 m²/g (for 57.00 ± 0.50 min) and foam stability in the range of 75–78%, respectively. The hydrolysates were able to stabilize drug and food formulations successfully and two of the samples exhibited less than 3% toxicity as determined through hemolytic assay.

Conclusion: Collagen hydrolysates from fish skin, a common fish industry waste, were found to be bioactive and non-toxic making them suitable replacements for synthetic emulsifiers.

Keywords: Fish waste; Collagen; Hydrolysate; Emulsifying agent; Food additives.

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Öz

Amaç: Hintli balık endüstrisi, her yıl bir kısmı kirliliğe neden olan depolama sahası olarak terk edilmiş olan şaşırtıcı miktarda atık üretmektedir. Bu çalışma, balık derisinden kolajen ekstraktının, bu atığın önemli bir bileşeninin çıkarılmasını, hidrolize edilmesini ve hidrolizatın gıda ürünlerinin raf ömrünü uzatmak için emülsiyonlaştırıcılar olarak kullanmayı amaçlamaktadır.

Gereç ve Yöntemler: Kolajen, Kılçık, Mullet ve Hint Somon derisinden saflaştırılarak infrared ve difraksiyon

çalışmaları yapıldı. Numuneler enzimatik olarak hidrolize edildi. Hidrolizatlar emülsiyonlaştırıcı, köpük stabilize edici ve hemolitik özellikler açısından analiz edilmiştir.

Tartışma: Örnekler, kolajen tip I'inkine karşılık gelen elektroforetik ve kızıl ötesi spektral profili gösterdi. X-ışını kırınım paternleri, üçlü sarmalın ve fosfat izlerinin varlığını ortaya koydu. Hidrolizatlar (6 kDa), en yüksek emülsiyonu sırasıyla% 75–78 aralığında $2.74 \pm 0.20 \text{ m}^2/\text{g}$ (57.00 ± 0.50 dakika) ve köpük stabilitesinde sergilemiştir. Hidrolizatlar, ilaç ve gıda formülasyonlarını başarılı bir şekilde dengeleyebildi ve iki örnek, hemolitik analiz yoluyla belirlenen% 3'ten az toksisite sergiledi.

Sonuçlar: Balık derisinden elde edilen kollajen hidrolizatlar, yaygın bir balık sanayi atığının, biyoaktif ve toksik olmadığı, sentetik emülgatörler için uygun yer değiştirmeler yaptığı görülmüştür.

Anahtar kelimeler: Balık atığı; Kollajen; Hidrolizat; Emülgatör; Gıda katkı maddeleri.

Introduction

India boasts of 7500 km long coastline, including 3 million hectares of reservoirs with access to high yielding fishing zones, making fish processing an exceptionally profitable trading commodity [1]. As per Government statistics, the fisheries sector exported 983,756 tonnes of processed products worth values 4.6 billion USD in 2013–2014 [2] and have contributed significantly to India's economic growth. The food processing industry of India is ranked fifth in terms of production, consumption, export and expected growth [3]. Depending on the level of processing and the type of fish involved, raw fish is subjected to a series of steps which gradually removes an average of 50% material from it, before being packaged for the final sale. The fish processing industry, as a consequence, ends up generating a huge amount of waste materials, part of which is conventionally utilized to form isinglass and animal/fish feed [4–6]. The extracellular protein collagen prevalent in these wastes has been reported to contain dormant fragments within their sequences, that when excised out, display myriad physiological activity [7–10]. Consequently collagen can be recycled from the waste, hydrolyzed to release bioactive fragments that can be utilized in emerging industries.

Existence of lipid based food is as primitive as early Bronze Age. Lipid based food are an amalgamation of

oil and water and displays a high tendency to separate leading to rancidity. This can be inhibited by the application of a group of organic moieties termed as emulsifiers which interacts simultaneously with both oil and aqueous phases aiding in the formation of a stable emulsion. Synthetic emulsifiers like polysorbate-80 and carboxymethylcellulose are considered to be in the “Generally Regarded As Safe” (GRAS) category and are routinely used in commercially available food products [11]. However, most substances in the GRAS category which are deemed non-toxic and non-carcinogenic have not been tested for any side effects upon continuous consumption. It has also been reported that these synthetic emulsifiers act like detergents leading to disruption of the mucous membranes of the intestinal walls with subsequent disruption of microbiome leading to increased inflammation [11]. Investigations have linked these synthetic additives to metabolic syndromes – including obesity, high blood pressure and type II diabetes [12].

Such impediments associated with synthetic emulsifiers have driven the current research towards natural emulsifiers that could reduce metabolic complications and provide health benefits to the consumer. A natural emulsifier needs to be non-immunogenic, non-toxic and should be able to display a level of activity that is equivalent to its synthetic counterpart. Collagen hydrolysate (CH) can be considered as a potential candidate for a natural emulsifier as it contains adequate amounts of both hydrophobic and hydrophilic residues and is known to be immunocompatible [13].

The present study attempts to assess the emulsification property of CHs obtained from Mullet, Catfish and Indian Salmon skin (henceforth referred to as MSK, CFSK and SLSK respectively), generally discarded as waste, so as to utilize them as food stabilizing agents.

Materials and methods

Materials

The chemicals used for the experiment were procured from various sources. Tris-HCl, N,N,N',N'-tetramethylethylenediamine, Glycine, Methanol, Ammonium Persulfate and Bromophenol Blue were procured from Merck Ltd., USA. The cellulose membrane and Coomassie Brilliant Blue was procured from HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India. All chemicals used for the assays were of analytical grade.

Collection of waste and Isolation of collagen

Skins of Mullet (*Mugil cephalus*), Catfish (*Heteropneustes teropfossilis*) and Indian Salmon (*Eleutheronema tertradactylum*) were collected from a fish market in Kumaraswamy Layout, Bengaluru, Karnataka. The skins were washed thoroughly with tap water and adhering muscles and bones were scraped off the skin using sterilized knife and forceps. Next, the dry weights of these skin samples were noted followed by fragmentation. Collagen was isolated from the fragmented pieces through acid dissolution and salt precipitation technique [10]. The details of the extraction process are displayed in the form of a flow scheme in Figure 1. Dry weight was noted after each step and the exact amount of collagen obtained with respect to the skin was calculated from the insoluble residue left after one complete round of extraction. The extracted collagen samples

were dialyzed against 0.005 M acetic acid and the dialyzed samples were freeze-dried.

Characterization of purified collagen

UV-Vis Spectra of collagen

The UV-Vis absorption spectrum of extracted collagen samples CFSK, MSK and SLSK were recorded in the range of 200–400 nm using a Shimadzu spectrophotometer UV-240.

Electrophoretic profile of Collagen

The purity of CFSK, MSK and SLSK was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 7% resolving gel against – rat

Figure 1: The process flow scheme outlines the protocol for collagen extraction.

Dry weight was noted after each step and the exact amount of collagen obtained with respect to the skin was calculated from the insoluble residue left after one complete round of extraction.

tail tendon collagen (RTTC) [14]. The gel was run for 2 h at 50 mV and on completion was stained with Coomassie Brilliant Blue for 30 min and de-stained till collagen bands were distinctly visible.

Fourier Transform Infra-red spectral analysis of collagen

Fourier Transform Infra-red (FT-IR) spectroscopy of lyophilized CFSK, MSK and SLSK were performed using a Bio-Rad FT-IR- 40 model, USA. Samples (2 mg) were mixed with 100 mg of dried potassium bromide (KBr), and compressed to form a salt disc (10 mm in diameter) for reading the spectrum.

X-ray diffraction pattern of collagen

X-ray diffraction (XRD) patterns of CFSK, MSK and SLSK were obtained using a Rigaku Ultimate IV machine with CuK α radiation from a rotating anode generator operated at 35 kV and 20 mA in the range of $2\theta = 10^\circ - 70^\circ$. The scanning step was 0.02° while the scanning speed was $2^\circ/\text{min}$. Peaks obtained were matched against standard XRD peaks of collagen.

Elution profile of collagen

An AKTA Prime Fast Protein Liquid Chromatography unit was used for obtaining sample elution pattern following standard protocol [15]. Individual samples (CFSK, MSK and SLSK) were loaded into a 1.5×13 cm Sephadex G100 column and elution was carried out at the flow rate of 1.0 mL/min. Absorbance was taken at 280 nm and fractions of 3 mL were collected. The column was calibrated using standard markers. The molecular weight of collagen samples were calculated by plotting their elution time in the standard calibration line. The purified collagen was stored to be used for hydrolysis.

Enzymatic hydrolysis of purified collagen samples

Collagenase type I, extracted from *Clostridium histolyticum*, was used to fragment the purified collagen samples. The enzyme was mixed with the substrate (intact collagen) in 0.1 M pH 7 phosphate buffer in the ratio 1:500 and the mixture was sealed at 37°C for 24 h. After the incubation

period, the mixture was heated to deactivate the enzyme and CH was extracted with 0.5 M acetate buffer.

Characterization of extracted CH

Electrophoretic band patterns were obtained by running the Catfish skin collagen hydrolysate (CFCH), Mullet skin collagen hydrolysate (MCH), Indian Salmon skin collagen hydrolysate (SLCH) samples in 10% SDS-PAGE gel along with a standard marker for molecular weight determination. Staining and destaining was done as before.

Bioactivity of CH

Emulsification assay

Emulsifying capacity was determined according to previous methods [16] using soya bean oil and 0.5 M phosphate buffer as the lipophilic and hydrophilic phases, respectively, with CH acting as the emulsifier. The emulsification activity and stability were calculated using the formula as given below.

$$\text{EAI} \left(\frac{\text{m}^2}{\text{g}} \right) = \frac{2.303 \times \text{Dilution factor} \times A}{c \times \theta \times 10,000 \times p}$$

Where, EAI is emulsification activity index, A is the absorbance at 520 nm; c is the protein concentration (g/mL); θ is the disperse phase volume fraction (0.25); and p is the optical path (0.01 m).

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

Where, ESI is the Emulsion Stability Index, A_0 is the absorbance at 520 nm at time 0 min; A_{10} is the absorbance at 520 nm at time 10 min; and t is the time difference. Poly ethylene glycol (PEG), a known emulsifier was used as the positive control (PC) while intact collagen sample was used as the negative control (NC).

Foaming stability

Foaming Stability was determined according to the previous methodology [17] using soya bean oil and 0.05 M phosphate buffer as the lipophilic and hydrophilic phases, respectively, with CH acting as the emulsifier. This emulsion was vortexed at 10,000 rpm for 1 min and the foam volume

was noted at 5th, 10th and 30th min. The foam stability index (FSI) was calculated using the formula given below:

$$\text{FSI (\%)} = \frac{\text{Foam Vol. after } t \text{ mins}}{\text{Initial Foam Vol.}} \times 100$$

PEG was used as the PC while intact collagen sample was used as the NC.

In vitro diffusion assay of CH

The CH samples were tested for the strength of the emulsion formed through an in vitro diffusion assay. Atorvastatin, a sparingly soluble drug was dissolved in methanol at a concentration of 150 mg mL⁻¹ and the volume was made up to 6 mL with phosphate buffer, pH 7. CH (2.5 mg mL⁻¹) was added in an equal volume ratio to the drug suspension and homogenized to create the emulsion. PEG and intact collagen were used as the positive and NCs, respectively. The emulsion was dispensed inside a dialysis bag, clamped and placed inside a dialysis chamber containing 800 mL 0.2% methanol as the dialysis medium. Aliquots of 5 mL were removed from the medium at 1 h interval, suitably diluted and checked for the drug by a UV-visible Spectrophotometer (Shimadzu 8400) at 244 nm. The absorbance was matched with a standard dilution series of the drug to find the amount diffused out. The medium was replaced with the same solution to maintain a constant volume. The % of diffusion was calculated by the following equation

$$\% \text{ Diffusion} = 100 \times \frac{\text{Test}}{450 \text{ mg}}$$

Where Test represents the amount obtained from the dialysis medium in mg. The assay was conducted in triplicates.

Hemolytic assay of CH

Hemolytic assay was performed as per standard protocol [18] with fresh blood sample. Hemolysis percentage was calculated using the formula given below:

$$\text{Hemolysis (\%)} = \frac{A_t - A_{nc}}{A_{pc}} \times 100$$

Where A_t is the absorbance of the test sample, A_{pc} and A_{nc} are the absorbance of PC and NC, respectively. Cells

incubated with Tween 20 were used as the PC while cells incubated with glycine were used as the NC.

Food emulsion stability assay

MCH was chosen for the food emulsion stability assay based on its efficient emulsion stability and low toxicity as seen in hemolytic assay. Jelly and coconut paste, two common household food items were chosen for the analysis. The food items were mixed with MCH (in phosphate buffer) at a ratio of 100:1. As control, only phosphate buffer was added to the food. Food mixed with intact collagen was taken as the NC and glycine – added food was taken as the PC. The samples were kept for 4 weeks at room temperature in a closed-lid container. Triplicates were used for each sample. A fixed volume of sample (500 µL) was aspirated every 24 h while maintaining sterile conditions. The aliquot was diluted with deionized water and absorbance read in a spectrophotometer. A second assay was performed in which the same set of samples were stored in the refrigerator for 4 weeks and emulsion stability was checked once a week as mentioned above.

Statistical assay

The emulsification, foam stability, drug diffusion, hemolytic and food stability assay were performed in triplicates. Values were checked with two way ANOVA and p-values <0.05 were considered as significant.

Results

Extraction and characterization of collagen

The extraction efficiency of collagen is shown in Table 1. As seen from the table, the total yield of collagen from fish skin was restricted to about 3–14% with highest amount (13.7%) extracted from MSK. Spectrophotometric analysis of the fish skin samples revealed a diminished peak at 280 nm and a significantly high peak at 220 nm which is a characteristic for collagen type I (not shown in figure). The SDS-PAGE pattern of CFSK and MSK displayed a double band of α_1 and α_2 , along with repeating β and γ bands of higher molecular weights, specific to collagen type I (Figure 2, lane 1 and 2). The band pattern matched with that of RTTC (lane 3) used as a standard to identify collagen type I. The molecular weight of the α_1 chain from

Table 1: Extraction efficiency of collagen from Catfish, Mullet and Indian Salmon.

Skin sample	W1 (g) dry weight	W2 (g)	W3 (g)	W4 (g)	Insoluble material W5 (g)	Soluble collagen (g) W5-W4	% Collagen (wt/g of skin) [(W5-W4)/W1] × 100
Catfish	48.8 NaOH	42.5 Butanol	30.5 EDTA	15.6 Acetic acid	13.65	1.95	3.9
Mullet	61.2	55.5	44.4	36.25	20.85	8.4	13.7
Indian Salmon	35.4	29.8	18.5	14.2	10.3	3.9	11.0

Figure 2: Electrophoretic profile of extracted collagen samples; CFSK (Lane 1), MSK (Lane 2) and positive control, rat tail tendon collagen (Lane 3).

The α_1 and α_2 bands along with higher order β and γ bands are visible.

collagen was found out to be 121.13 kDa, while the α_2 chain value was 117.51 kDa.

The FT-IR spectrum of CFSK, MSK and SLSK, as displayed in Figure 3, depicted multiple peaks corresponding to the presence of collagen. The peak details and their corresponding bond vibrations are given in Table 2.

Figure 3: FT-IR bands of collagen samples CFSK, MSK and SLSK. Along with typical peaks from collagen, minor peaks of phosphate were also observed.**Table 2:** FT-IR Peaks and their corresponding functional groups.

Peaks (cm ⁻¹)	Vibrations
1038	P-O asymmetric stretching
1240	Amide III – NH stretching
1337	CH ₂ wagging of proline
1556	Amide II – NH stretching
1634	Amide I – C=O stretching
2786	Amide B – CH ₂ symmetrical stretch
3362	Amide A – NH stretch coupled with hydrogen bonding

XRD was used to investigate the collagen fibril distribution. Figure 4A and B shows the X-ray spectrum of the lyophilized MSK and SLSK, respectively. The following peaks could be distinctly identified in both samples: 110 (18.82°), 200 (21.76°), 111 (22.85°), 211 (31.7°), 112 (32.8°), 300 (32.9°), 311 (41.99°), 312 (48.07°), 402 (52.07°) and 004 (53.17°).

The CFSK, MSK and SLSK samples eluted with a minor peak at 7.5 ± 1.5 min and a major peak at 19 ± 0.2 min in sephadex G100, as exhibited in Figure 5, indicating the collagen to be of type I. The molecular weight of the major peak was found out to be 121 ± 3.5 kDa, by correlating with known markers eluted through the column.

Figure 4: X-ray spectrum data of the lyophilized collagen from (A) Mullet and (B) Salmon skin.

Major peaks correspond to the presence of collagen along with minor amounts of hydroxyapatite.

Electrophoretic profile of CH

Hydrolysis was confirmed with the absence of higher order bands and the presence of low molecular weight bands as displayed in Figure 6. A graph was plotted with molecular weights of the marker against distance moved and the hydrolysates were found to exhibit a molecular weight of 6 kDa.

Bioactivity of CH

Emulsification assay of CH

The results of the emulsification assay are displayed in Figure 7A and B. The EAI and ESI of CH were calculated

Figure 5: Gel permeation chromatography of collagen samples showing a minor and major peak for the aggregated polypeptides (β band) and individual α chains, respectively.

Note the slight higher absorbance for CFSK indicating presence of more tyrosine residues when compared to SLSK and MSK.

Figure 6: SDS-PAGE of collagen hydrolysates (Lane 1: MCH, Lane 2: CFCH, Lane 3: SLCH, Lane 4: RTTC hydrolysate) run against standard marker in Lane 5.

from difference in absorbance at 0th min and 10th min. At low concentrations (0.005%), MCH and CFCH displayed similar emulsifying ability, followed by SLCH. However, at a higher concentration (0.05%), MCH displayed significantly high ($p < 0.05$) emulsion stability when compared to CFCH and SLCH.

Figure 7: The CH samples displayed moderately strong emulsifying activity and high emulsion stability time. (A) EAI and (B) ESI activity exhibited by CH samples, with intact collagen as NC and PEG as PC.

All three samples displayed similar ESI at low concentrations and the time duration of emulsion stability (30 min) was about 50% that of the PC. However, at high concentration (0.05%), CFCH could stabilize the emulsion for more than 50 min, which was significantly higher ($p < 0.05$) than SLCH and MCH, both of which displayed similar stability time of 22–25 min. Interestingly, the PC exhibited significantly lower ESI at a concentration of 0.05%, possibly because of low solubility and miscibility.

Overall, the CH samples displayed significantly higher EAI and ESI than the intact collagen, used as the NC.

Foam stability assay of CH

It was found that the CH could significantly ($p < 0.05$) stabilize foam volume when compared to the intact collagen sample (MSK) as shown in Figure 8. All three samples CFCH, MCH and SLCH (at 0.05%) displayed retention of 75–78% of foam volume after 30 min.

Figure 8: FSI value of CH with intact collagen (MSK) for comparison.

In vitro diffusion assay of CH

As exhibited in Table 3, the rate of diffusion of the drug from the emulsion was significantly lowered ($p < 0.05$) by the addition of CFCH. Interestingly, the rate was analogous to that of the diffusion rate from PEG emulsion, indicating an emulsion stability equivalent to a known emulsification agent.

Toxicity assay of CH

As observed from Figure 9, the hemolysis percentage of CFCH, MCH and SLCH samples at concentration of

Table 3: Results of the drug diffusion assay.

Time (h)	% Diffusion		
	Free drug	PEG	CFCH
0	0 ± 0	0 ± 0	0 ± 0
1	0.4 ± 0.6	0 ± 0	0 ± 0
2	2.1 ± 0.4	0 ± 0	0 ± 0
3	4.8 ± 0.2	1.5 ± 0.7	0 ± 0
4	9.5 ± 1.2	2.2 ± 0.3	0 ± 0
5	18.4 ± 2.7	3.1 ± 0.3	1.4 ± 0.2
6	32.0 ± 3.5	3.5 ± 0.4	3.9 ± 0.3
7	48.0 ± 2.0	4.8 ± 1.5	5.6 ± 0.3
8	54.0 ± 2.6	5.1 ± 0.9	6.8 ± 0.4

The columns (from the left) indicate the % of drug leaching out of the emulsion for the free drug, PEG (used as PC) and CFCH.

Figure 9: Hemolytic activity of CH at a concentration of 2.5% with cells incubated with glycine and Tween 20 as NC and PC, respectively.

2.5% (w/v) was 12.40 ± 1.01 , 1.08 ± 1.06 and $2.77 \pm 0.85\%$, respectively.

Food stability assay

The activity of the CH samples on food stability was analyzed in both refrigerated and non refrigerated condition. As seen in Figure 10A–D, jelly mixed with intact collagen (used as NC) and glycine (used as PC) displayed increasing absorbance indicating enhanced breakdown of the emulsion. However, the food mixed with MCH showed a constant absorbance indicating a stable emulsion for 4 days in non refrigerated condition. Similar results were obtained with refrigerated experimental set-ups. The emulsion and texture of the CH mixed food was found to be stable for up to 4 weeks while other samples displayed a gradual loss in stability.

Figure 10: Food stability assay results with MCH as the test sample, phosphate buffer as blank-B, glycine as PC and MSK as NC. The emulsions tested were (A) coconut *chutney*, represented as CC, kept at room temperature (B) CC kept at 4°C, (C) jelly, represented as J kept at room temperature and (D) J kept at 4°C. The MCH displayed emulsion stability equivalent to that of PC.

Discussion

Extraction and characterization of collagen

As displayed in the process flow scheme, collagen is extracted by treating the skin samples with NaOH, 10% butanol and EDTA to remove non-collagenous proteins, fat and minerals, respectively. The resultant residue is subjected to acetic acid dissolution to obtain pure acid-soluble collagen. The % collagen obtained in the study (3.9–13.7%) was lower than that obtained from Egyptian Nile Tilapia (*Oreochromis niloticus*) scales [19] and Yellowfin tuna (*Thunnus albacares*) skin [20] which yielded 23.0 and 27.1% collagen, respectively, and similar to that of jumbo squid (*Docidicus gigas*) [21] and big-eye snapper (*Priacanthustayenus*) [22] which yielded 5% and 11% collagen, respectively. The difference in collagen yield obtained in this study was due to the extraction method administered and the variation in quality, age and type of fish [23].

The electrophoretic profile of the extracted collagen samples exhibited a double band at 121.13 and 117.51 kDa in accordance with the relative arrangement of α chains in collagen type I, the most abundant type of collagen in skin. Each tropocollagen unit of collagen type I comprises of a triple helix consisting of two α_1 chains and one α_2 chain [$(\alpha_1)_2(\alpha_2)_1$]. This unequal distribution pattern of α chains leads to α_1 (121.13 kDa) displaying a stronger band intensity than that of α_2 (117.51 kDa) [24]. The high recoiling tendency of collagen α chains forms inter chain associations resulting in high molecular weight β and γ bands.

FT-IR studies reveal specific vibration bands characteristic of peptide bonds and side chain groups. The isolated samples displayed the band regions characteristic to that of collagen, including Amide I, II, A, B and C [25–27]. The XRD peaks showed that this protein had ordered structure snippet, confirming presence of stable triple helical collagen fibril [28, 29]. However, both XRD and FT-IR data revealed that the collagen sample was contaminated with residual phosphate group. Hence, the collagen was purified by running in gel permeation column before converting it into hydrolysate.

The major gel permeation elution peak, as shown in Figure 5, was due to the presence of non-aggregated α chains in the molecular weight range 118–125 kDa. The minor initial elution peak is a result of the aggregation of α_1 and α_2 polypeptide chains to form the β and γ bands. Similar elution patterns have been observed for collagen extracted from big eye snapper [22] and skin of tilapia [24]. Even though equal amounts of the collagen samples were run in FPLC, CFSK displayed a higher absorbance peak

indicating presence of comparatively higher tyrosine and tryptophan (hydrophilic aromatic residues) with respect to the other two collagens – SLSK and MSK.

Activity of CH

Protein hydrolysates, including that of collagen are surface-active materials and promote an oil-in-water emulsion because of comparatively higher abundance of hydrophobic groups [30, 31]. Fragmentation of protein also increases the number of exposed N- and C-terminals consequently increasing the hydrophilicity of collagen. This provides a balance between abundance of hydrophobic and hydrophilic residues allowing the CH to act as an effective emulsifier.

The samples CFCH and MCH showed better EAI when compared with SLCH at 0.05%. All three samples displayed EAI almost equal to that of eel skin gelatin, –63.97% at 2% concentration [32] but less than Spanish mackerel CH $-97.44 \pm 1.76\%$ at 1% concentration [33]. All three CH samples could retain >75% foam volume for over 30 min, which was significantly better than Spanish mackerel CH with a reported 65.27% foam retention [33].

The three samples, CFCH, MCH and SLCH exhibited better ESI and FSI when compared to EAI, which indicated that the CH could stabilize low emulsion/foam volumes but for a longer duration when compared to a standard emulsifier. The interesting thing was that even though all three samples contained collagen type I fragments, differences in activity were noticeable. This is because, even though collagen type I is conserved throughout the animal kingdom, minute discrepancies in sequences do exist based on the type of fish. The number of cross links will also be more in older fishes leading to lower availability of specific sequences. These events lead to change in the hydrophilic to hydrophobic residue ratio in fish-extracted collagen resulting in slight changes in emulsion stabilization properties. Another interesting pattern that could be traced is that the activity of hydrolysates did not entirely correlate with its concentration. The lowering of activity at higher concentrations in most cases was due to the fact that at higher concentrations, the peptides of a CH exhibits a tendency to coil with each other [10, 15], thus effectively reducing its interaction with both aqueous and oil phases. This tendency increases with increasing abundance of hydrophobic residues in the CH, as observed for MCH and SLCH. However, the effect was not observable in CFCH, possibly because of slightly lower amount of hydrophobic residues and higher hydrophilic residues as indicated by the increased absorbance during elution (Figure 4).

To summarize, the amount of CH used in a particular emulsion is of paramount interest – it should not be too low, lest it does not form a stable emulsifier nor too high so that it may recoil and destabilize the emulsion.

The diffusion assay revealed that the CFCH could successfully maintain the drug emulsion with the lowest value of 6.75% leakage in 8 h. Thus the samples could be used as drug encapsulation agents or time capsules that would stabilize a drug in vivo and initiate controlled release, increasing its bio availability. The food stability assay was performed using a spectrophotometer to monitor the stability of the emulsion formed by MCH over a fixed time period. The CH was able to sustain the emulsion as long as the PC, substantiating the earlier results obtained with soya bean oil and in the diffusion assay. Both assays confirm the unsurpassable potential of skin CH as an emulsifying agent.

The Food and Drug Administration mandates that every compound added to food has to be tested for toxicity. The samples SLCH and MCH displayed less than 3% erythrocyte breakdown after 30 min of incubation with whole blood, proving their low hemolytic tendencies.

Conclusion

Hydrolysate-based emulsifiers are a promising alternative over their chemical counterparts due to their high efficiency, biodegradability and low toxicity. Based on the relative ease of extraction and the high emulsion/foam stability of CH as seen in this study, it can be concluded that Mullet and Indian Salmon skin are safe to be used as food stabilizing agents.

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