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Isolation of acid soluble collagen from daggertooth pike conger (*Muraenesox cinereus*) and evaluation of its antimicrobial activity#

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Abstract

The study was conducted to isolate acid soluble collagen (ASC) from daggertooth pike conger eel and to evaluate the antimicrobial activity of the isolated protein. Conger eels were collected from Kochi harbour. The proximate composition of eel skin consisted of 68.73 per cent of moisture on a wet basis with a crude protein, crude fat and total ash of 30.24 per cent, 12.17 per cent and 2.16 per cent respectively on a dry matter basis. Conger eel skin collagen was isolated by treating with 0.5 M acetic acid after pretreatment with 0.1 N NaOH and 10 per cent butanol. Salting out and precipitation of ASC was carried out by adding NaCl to a final concentration of 2.3 M. Precipitated ASC was then dialysed against 0.1 M acetic acid for 24h and then in distilled water until neutral pH was obtained. The yield of ASC was 12.78 per cent on a wet basis and 31.95 per cent on a dry basis. The antimicrobial activity of ASC was determined by the agar diffusion method. ASC showed antimicrobial activity against both E. coli and S. aureus.

Keywords: *Daggertooth pike conger, acid soluble collagen, agar diffusion method*

Collagen is the most abundant fibrous structural protein in the extracellular matrix. Collagen is characterised by a triple helix structure composed of three alpha subunits (Gelse *et al.,* 2003). So far, 29 different types of collagens have been identified (Lin *et al.,* 2019). Collagen has traditionally been extracted from the skins of land animals like cows and pigs. Collagen derived from bovine sources showed appreciable wound healing activity and act as an excellent graft in cutaneous wounds (Vishnu *et al.,* 2016). Additionally, collagen can also be obtained from avian

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species, especially from the skin of turkey due to its high concentration of collagen (Sona *et al*., 2020). The use of collagen and collagen-derived products from land animals has drawn increased attention in recent years due to the spread of transmissible diseases from animal origin (Karim and Bhat, 2009). In their quest to discover another collagen source, the researchers turned their focus to the world's oceans.

Fish skin is a frequently available, inexpensive material with high biological value and high essential amino acid content. The structural analysis and gene sequencing results revealed that collagen from marine sources had a high denaturation temperature and maintained its distinctive triple helix shape even in the presence of environmental perturbations (Miles *et al.,* 2005). Collagen has a wide range of uses in the cosmetic, pharmaceutical, biomedical, film, and leather sectors (Sionkowska *et al*., 2017). Because of its distinctive qualities, including biodegradability and mild antigenicity, soluble collagen is beneficial in a variety of industries, including food, cosmetics, and pharmaceuticals (Gomez *et al.,* 2011).

The identification and characterization of collagen type I from daggertooth pike conger eel are mentioned in this research work (*Muraenesox cinereus*). The Muraenesocidae family of pike conger eel, includes the marine fish known as conger eel, which has an annual harvest of more than 340,000 tonnes (Dou, 2014). The use of the acid soluble method for extraction offers benefits including the need for few resources (equipment and time), high productivity with little waste, and low manufacturing costs. The present study was conducted to extract acid soluble collagen from these conger eels and evaluate the isolated protein's antimicrobial activity.

Materials and methods

Sample collection and initial treatment of fish skin

The Daggertooth pike conger (*Muraenesox cinereus*) was collected from fish landing centres at Kochi, Kerala, India (9°55'52.44"N, 76°16'2.29"E) and stored at -20°C. The outer skin was removed and washed using cold distilled water. The residual meat was removed carefully. The obtained skin was sliced into small pieces (1.5±0.5 cm^2), rinsed in cold water and packed in polythene bags. Prepared skin was stored at -20°C until collagen extraction.

Proximate composition

The proximate composition of initially treated fish skin was determined. The content of moisture on a wet basis and crude protein, crude fat, and total ash on a dry matter basis were analysed by AOAC (2016) method.

Pre-treatment of outer skin for the removal of fat and non-collagenous protein

Acid soluble collagen (ASC) was extracted from eel skin following the protocol of Nagai and Suzuki (2000) with slight modification. All the extraction techniques were carried out at 4°C. The skin pieces were kept in 10 per cent butyl alcohol solution for 48 h at a sample-to-alcohol ratio of 1:10 (w/v) for defatting the skin. Defatted skin was washed using ice-cold distilled water. The non-collagenous protein was removed by treating the defatted skin with 0.1 *N* NaOH solution at a sample-to-solution ratio (w/v) of 1:10 to remove non-collagenous proteins. The alkali treatment was for three days, changing the solution every 24 h and the solution being stirred at eight-hour intervals. After the treatment, the samples were washed until neutral pH was obtained and freeze-dried.

Extraction of acid soluble collagen

The deproteinated skin was then soaked in 0.5 *M* acetic acid at a sample-to-acid ratio of 1:15 (w/v) for three days at 4°C with gentle stirring. The supernatant was collected from the extract after centrifugation at 20,000 × *g* for one hour and kept at 4°C. The residue was re-extracted as above by 0.5 *M* acetic acid treatment for two days. The combined supernatant from both extracts was mixed and taken as acid soluble collagen.

Salting out and dialysis

Collagen from the viscous solution was salted out using sodium chloride to a final concentration of 0.9 *M*. Precipitation of collagen was carried out by the addition of NaCl to give a final concentration of 2.3 *M* in 0.05 *M* Tris-HCl (pH 7.5). The resultant precipitate obtained was further centrifuged at 20,000× *g* for 20 min at 4°C. The pellet dissolved in 10 volumes of 0.5 *M* acetic acid dialysed against 10 volumes of 0.1 *M* acetic acid for a day followed by distilled water until a neutral pH was obtained and lyophilised using Operon FDU 7003 lyophiliser.

Yield of extracted acid soluble collagen

The yield of ASC was determined by measuring the percentage weight of freeze-dried collagen to the weight of the skin on both a dried and wet basis.

$$\text{Collagen (\%)} = \frac{\text{Weight of lyophilised collagen in grams} \times 100}{\text{Weight of skin in grams (dry weight)}}$$

$$\text{Collagen (\%)} = \frac{\text{Weight of lyophilised collagen in grams} \times 100}{\text{Weight of skin in grams (wet weight)}}$$

Antimicrobial activity

The antibacterial activity of isolated ASC against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* was determined by agar diffusion assay. Standard bacterial cultures were procured from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh. The standard cultures of both strains were grown in a Luria broth liquid medium. The inocula from the liquid media were spread on to Luria broth agar plate. Collagen with concentrations of 25, 50, 75 and 100 µg were inoculated into sterile discs. These were placed into the agar plate. Alongside streptomycin 25 µg antibiotic disc was also placed. The plates in triplicates were kept in the incubator for 37°C. The zones of inhibition were measured after 18 h of incubation. Diffusion distances were calculated and examined separately to estimate MIC, and the final MIC was calculated using the average results from three replicates.

Statistical analysis

All the analysis and extraction technique of acid soluble collagen from conger eel was replicated thrice and expressed as mean ± SD.

Results and discussion

Daggertooth pike conger eels were collected from the harbours of Kochi. The skin was sliced and kept at -20°C until it was used for further analysis. Similar temperature conditions are mentioned by Muyonga *et al.* (2004) for the extraction of ASC from Nile perch.

Proximate composition

The skin from daggertooth pike conger eel has the proximate composition (Table 1) of moisture of 68.73 ± 0.23 per cent on a wet matter basis and crude protein, crude fat and total ash of 30.24 ± 0.18, 12.17 ± 0.07 and 2.16 ± 0.03 per cent, respectively on dry matter basis. When compared to conger eels, the marine eel *Evenchelys macrura* had slightly higher moisture, total ash, and crude protein compositions of 75.89, 8.82, and 90.05 per cent, respectively and lower crude fat composition of 1.23 per cent (Veeruraj *et al.,* 2013). Similarly, the collagen from Nile perch contained 68.4 per cent moisture similar to pike conger eel, and lower protein per cent of 21.6 and fat of 6.8 per cent and higher ash per cent of 6.0 (Muyonga *et al.,* 2004).

The moisture content of pike conger eel was also similar to that of the blacktip shark (67.12 %) and lower than that of brown-backed toadfish (73.14 %) (Kittiphattanabawon *et al.,* 2010; Senaratne *et al.,* 2006). According to Veeruraj *et al.* (2015), the crude protein content of squid fish skin was found to be 31.79 per cent, which is comparable to the crude protein of conger eels. Similarly, the crude fat per cent was higher compared to brown banded bamboo shark fish and brown backed toadfish where the fat per cent obtained 0.19 and 1.3 respectively (Kittiphattanabawon *et al.,* 2010; Senaratne *et al.,* 2006). The proximate composition of fresh skin from hammerhead sharks showed slightly higher ash and lipid content of about 25.68 and 14.59 per cent and lower protein and moisture

content of about 18.88 and 40.85 when compared with conger eels (Chi *et al*., 2014).

Table 1. Results of proximate analysis of raw eel skin

On wet basis		On dry matter basis	
Moisture	68.73%	Crude protein	30.24%
		Crude fat	12.17%
		Total Ash	2.16 %

Isolation of acid soluble collagen

The protocol described by Nagai and Suzuki (2000) was followed for the pretreatment of the skin and for the extraction of ASC with slight modifications. The same pretreatment method to remove the fat and non-collagenous protein and a comparable acid extraction technique to isolate ASC were adopted in squid (Veeruraj *et al.,* 2015) and in marine eel *Evenchelys macrura* (Veeruraj *et al.,* 2013). Salting out, precipitation and dialysis of ASC extracted from conger eel was carried out by NaCl to a final concentration of 2.3 *M*. The precipitated proteins were dialysed until a neutral pH was obtained. A similar procedure was adopted by Chi *et al.* (2013) for salting out of ASC from hammerhead sharks, in which precipitation took place at a final concentration of 2.6 *M* whereas salting out of ASC from the skin of silver catfish was observed at a final concentration of 0.7 *M* NaCl (Hukmi and Sarbon, 2018).

Yield of collagen

The yield of ASC after acid extraction was 12.78 ± 0.11 per cent on a wet basis and 31.95 ± 0.14 per cent on a dry weight basis. The difference in the yield of ASC might be due to differences in collagen composition, structure, biological conditions during extraction, preparation methods and finally difference in species of fish used (Duan *et al.,* 2012). During acid extraction, the skin was not completely solubilized in 0.5 *M* acetic acid. This decrease in solubility might be due to intermolecular cross-links and covalent crosslinks formed by the condensation of the aldehyde group in the telopeptide region (Singh *et al.,* 2011). The yield of ASC obtained from marine eel was 80 (dry basis) and 9 per cent (wet basis), which was higher compared to conger eels (Veeruraj *et al.,* 2013). The yield of ASC from brown stripe red snapper, bigeye snapper, and large fin long barrel was 13.7, 7.5 and 28 per cent on a dry basis, respectively (Jongjareonrak *et al.,* 2005a; Jongjareonrak *et al.,* 2005b; Zhang *et al.,* 2009).

Antimicrobial activity

The antimicrobial activity of various concentrations of collagen is depicted in Fig.1 and Table 2 below. Collagen demonstrated antimicrobial activity against the strains of *S aureus* and *E coli*. The zone of inhibition obtained for collagen towards *E coli* was 120 mm for 100 µg. In the case of *S aureus* zone of inhibition was 110 mm for 75 µg and 140 mm for 100 µg. The antimicrobial activity of collagen might be due to the presence of collagen peptides present in it. Cationic amino acid residue in the collagen molecule interacts with the bacterial cell membranes, causing an increase in membrane permeability and thereby resulting in the lysis of the bacteria (Lima *et al*., 2015). Similar results were reported by Palanivel *et al.* (2019) and Shalaby *et al*. (2019), who noticed a zone of inhibition for collagen extracted from the skin of snakehead murrel and fish scales. The proline residues in the fish collagen also possess antimicrobial activity. These residues kill bacteria through a non-lytic process in which they pass through the outer membrane, penetrate the inner membrane using protein-mediated transport, and then target the ribosome to prevent protein synthesis (Reddy *et al.,* 2004).

Table 2. Antimicrobial activity of collagen gel against various bacterial strains

Strain	Zone of inhibition at different concentrations				
	25µg	50 µg	75 µg	100 µg	Positive control (Streptomycin)
S aureus	-	-	110mm	140 mm	210 mm
E coli	-	-	-	120 mm	200 mm

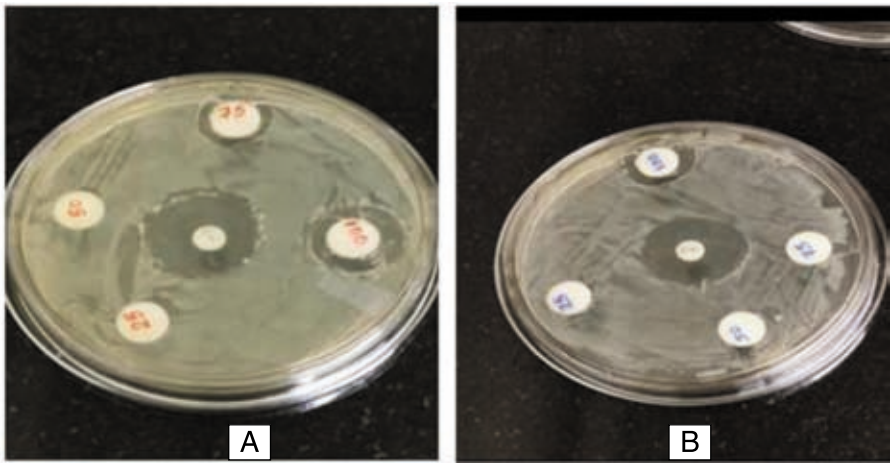

Fig.1. Antibacterial activity of acid soluble collagen against (A) *S aureus* was obtained at 75 µg and 100 µg; (B) *E coli* obtained at 100 µg concentration

Conclusion

The present study concluded that collagen could be successfully isolated from the skin of conger eel by dissolving it in acetic acid. It was observed that maximum precipitation of collagen protein was obtained at a final NaCl concentration of 2.3 *M*. Yield of ASC was 12.78 per cent on a wet basis and 31.95 per cent on a dry weight basis. The proximate composition of eel skin was estimated. Extracted fish skin collagen was tested against the two pathogenic bacteria i.e, *Escherichia coli and Staphylococcus aureus.* It was demonstrated that ASC demonstrated a zone of inhibition against both the strains at 100µg concentration, indicating the antimicrobial activity of ASC.

Conflict of interest

The authors declare that they have no conflict of interest.

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