Detection of collagen through FTIR and HPLC from the body and foot of *Donax cuneatus* Linnaeus, 1758

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Abstract To make more effective use of available marine resources, acid soluble collagen (ASC) was isolated from body and foot of wedge clam *Donax cuneatus* Linnaeus, 1758 with acetic acid and was characterized for their potential and commercial applications. The yield of ASC was 17% and 23% respectively. SDS PAGE, UV and FTIR spectroscopy showed that both were type I mainly with slight differences. HPLC was used for identifying the presence of different types of amino acids, where glycine was more or less 20% in both the samples and takes the lead amino acid position and presence of imino acids (11.8 and 12.6%) has been the characteristic feature of type I collagen.

Keywords Collagen · *Donax cuneatus* · FTIR · HPLC · Imino acids

Introduction

Collagen is one of the most abundant animal proteins, constituting approximately one-third of the total body protein

of mammals. There are at least 27 different types of collagen, named type I–XXVII (Birk and Bruckner 2005). Type I collagen is a right-handed triple superhelical rod consisting of three polypeptide chains and is found in connective tissues, including tendons, bones and skins (Muyonga et al. 2004). Commercially collagen has been isolated from the skin of terrestrial mammals like cow, pig and widely used in food, cosmetic, biomedical and pharmaceutical industries (Ogawa et al. 2004). But, the outbreak of highly infectious and contagious diseases such as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and food and mouth disease (FMD) in pigs and cattle has limited the use of collagen derived from them. Therefore, many scientists have been focusing to find out alternative sources for collagen and they found different organisms from marine source such as black drum (*Pogonia cromis*) (Ogawa et al. 2003), brownstripe red snapper (*Lutjanus vitta*) (Jongjareonrak et al. 2005), ocellate puffer fish (*Takifugu rubripes*) (Nagai et al. 2002) and leather jacket (*Odonus niger*) (Muralidharan et al. 2011). However, collagen from freshwater fish, were rarely reported, except for the Nile perch (*Lates niloticus*) (Muyonga et al. 2004), grass carp (*Ctenopharyngodon idella*) (Zhang et al. 2007) and channel catfish (*Ictalurus punctatus*) (Liu et al. 2007). Moreover bivalve (Nazeer et al. 2011) and gastropod (Nazeer and Srividhya 2011) has been proved the availability of medicinally important products. Therefore, the above investigations are showing scope for the identification of collagen like medicinal products from the useful and underutilized body parts of abundant coastal bivalve *Donax cuneatus*.

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Materials and methods

Collection of sample

Donax cuneatus Linnaeus, 1758 was collected from Kovalam sea coast (Lat. 12°47'12"N Lon. 80°15'01"E), Chennai, Tamil Nadu, India. The specimen was confirmed after thorough identification using the basic key characters (Shanmugam et al. 1998). The samples were washed thoroughly with running water and packed in polythene bags kept in ice box then brought to laboratory. The collected samples were dissected and cut into small pieces and stored at −20°C until used.

Isolation of collagen

The collagen was extracted according to the method of Nagai and Suzuki (2000). All the following processes were carried out at 4°C. Body and foot collected from *Donax cuneatus* were soaked in 0.1 M NaOH for 24 h with gentle stirring. The solution was changed every 8 h to remove non collagenous proteins and pigments. Alkali-treated samples were then washed with distilled water and defatted with 10% (v/v) butyl alcohol for 24 h with gentle stirring and a change of solution every 8 h. Defatted organs were thoroughly washed with distilled water. The matter was soaked in 0.5 M acetic acid with a ratio of 1:30 (w/v) for 24 h with gentle stirring. The mixture was then centrifuged at 9,000 g for 1 h at 4°C. The supernatants were collected and dialyzed with 10 volumes of 0.1 M acetic acid in a dialysis membrane for 12 h with change of solution for every 4 h. Subsequently, the solution was dialyzed with 10 volumes of distilled water with changes of water until neutral pH was obtained. The dialyzed solution was freeze-dried and used for further studies.

SDS polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the method of Laemmli (1970) using the discontinuous Tris–HCl/glycine buffer system with 7.5% resolving gel and 4% stacking gel. The collagen sample was dissolved in a sample buffer (0.5 M Tris–HCl, pH 6.8, containing 2% SDS, 25% glycerol) with 10% β -mercaptoethanol, to reach a final collagen concentration of 1 mg/ml, and then boiled for 3 min. The gel was stained for 30 min with 0.25% coomassie brilliant blue solution and destained with 7.5% acetic acid/5% methanol solution until the bands were clear.

Fig. 1 SDS-PAGE patterns of ASC from the body and foot of *Donax cuneatus* on 7.5% gel. Lane 1 ASC foot; lane 2 ASC body; lane 3 high molecular protein marker

UV spectroscopy

The ultraviolet absorption spectra of extracted collagens were recorded individually by a spectrophotometer. The collagen samples were prepared by dissolution in 0.5 M acetic acid solution with a sample/solution ratio of 1:1000 (w/v) (Wang et al. 2007).

Fourier Transform Infrared Spectroscopy (FTIR)

Infrared spectra from 4000 to 400 cm^{-1} were obtained with KBr disc method using an infrared spectrophotometer (Seprum RXI, Perkin Elmer). The number and location of amide band were provided by Fourier self-deconvolution, which was conducted using a resolution enhancement factor of 2.1 and half-height bandwidth of 13.5 cm^{-1} (Wang et al. 2007).

Fig. 2 UV spectra of ASC from foot and body of *Donax cuneatus*

Amino acid analysis

Amino acid analysis was performed using HPLC by the method of Huesgen (1998). The collagen extracts were dissolved in 6 N HCl and were subjected to hydrolysis in boiling water bath at 110°C for a period of 24 h. The tubes were cyclo-mixed for every 1 h for proper hydrolysis to take place. After 24 h of hydrolysis, the tubes were centrifuged at 3500 rpm for 15 min. The supernatant was filtered and neutralized with 1 N NaOH. Then the filtered solution was diluted to 1:100 of volume (1 ml diluted to 100 ml) with milli-Q water and loaded onto High performance liquid chromatography (HPLC). HPLC analysis was carried out in an Agilent 1100 assembly system after precolumn derivatization with o-phthalaldehyde (OPA). Each sample (1 µl) was injected on a Zorbax 80 A C₁₈ column at 40°C with

detection at 338 and 262 nm. Mobile phase A was 7.35 mM/L sodium acetate/triethylamine (500:0.12, v/v), adjusted to pH 7.2 with acetic acid, while mobile phase B (pH 7.2) was 7.35 mM/L sodium acetate/methanol/acetonitrile (1:2:2, v/v/v). The amino acid composition was expressed as percentage of protein.

Results and discussion

Extraction of collagen

Defatted body and foot of *Donax cuneatus* were hardly soluble in acetic acid and the yield of the isolated collagen after salt precipitation at neutral pH was 17% and 23% respectively. This yield is higher than that of diamondback squid (1.3%) (Nagai 2003) but when compared with other

Fig. 3 FTIR analysis of collagen from body of *Donax cuneatus*

marine fishes like yellow sea bream (40%) (Nagai and Suzuki 2000) and brown backed toad fish (54.3%) (Senaratne et al. 2006) yields were very less.

SDS poly acrylamide gel electrophoresis (SDS–PAGE)

The SDS-PAGE patterns of ASC body and foot were analyzed and it was found that both body and foot collagen consisted of two α -chains ($\alpha 1$ and $\alpha 2$) as the major constituents as shown in Fig. 1. The patterns were analyzed basing on their molecular weight using a high molecular weight marker (Genei) as reference and they were similar to that of the type I collagen, and also in accordance with those of collagens from most other fish species previously reported (Giraud Guille et al. 2000; Nagai et al. 2001; Muyonga et al. 2004). It was suggested that both body and foot ASC isolated from *Donax cuneatus* were probably type I collagen, which consisted of two $\alpha 1$ chains and one $\alpha 2$ chain. However, it cannot be determined whether $\alpha 3$ -chain exists in the collagens, since $\alpha 3$ -chain has a migration similar to that of $\alpha 1$ -chain and it cannot be separated from $\alpha 1$ -chain under the electrophoretic conditions employed.

UV-Vis spectroscopy

As depicted (Fig. 2), the distinct absorbance of the collagen was obtained near 220 nm, which is contributed by transition of C = O in the peptide bond. Generally, tyrosine and phenylalanine are sensitive chromophores and show maximum absorbance at 283 and 251 nm, where ASC has no evident absorbance. The ultraviolet spectrum of both body and foot ASC had absorptions at 233 nm. This was in agreement with those of collagens from bullfrog skin (236 nm) (Li et al. 2004) and channel catfish skin (232 nm) (Liu et al. 2007). Therefore, acid-soluble collagen from body and foot of *Donax cuneatus* supports the property of collagen by having its absorbance at 220–230 nm, with low absorbance at 280 nm. Thus the extracted protein is collagen.

Fourier Transform Infrared Spectroscopy (FTIR)

The infrared spectra of collagen from body and foot of *Donax cuneatus*, major peaks with their assignments are shown in Figs. 3 and 4. The infra red spectra of collagen from body and foot have differed slightly, indicating some

Fig. 4 FTIR analysis of collagen from foot of *Donax cuneatus*

Table 1 FTIR spectra peak locations of collagen from body and foot of *Donax cuneatus*

^a Body	^a Foot	Region
3429	3411	Amide A- NH stretch coupled with Hydrogen bond
2924	2960	Amide B- CH ₂ Asymmetrical stretch
1650	1645	C = O stretch
1235	1242	NH bend coupled with CN stretch
1120	1122	C-O stretch

^a—Peak wave number (cm⁻¹)

differences in the secondary structure of the collagen. And the triple helical structure of body and foot were conformed from the transmission ratio of 1235 and 1242 cm⁻¹ (amide III) band, which was approximately equal to the reported data (Plepis et al. 1996). The amide A band is associated with N-H stretching frequency but, according to Doyle et al. (1975), a free N-H stretching vibration occurs in the range 3400–3440 cm⁻¹ the amide A band of body and foot were at 3429 and 3411 cm⁻¹ respectively which shows correlation with present study. The peaks of amide I and II of body and foot were showing slight difference with each other as shown in Table 1. These indicated a presence of higher degree of molecular order, since the shift of these peaks to higher frequencies was associated with an increase in the molecular order (Payne and Veis 1988).

Amino acid analysis

The amino acid composition of acid-soluble collagens of *Donax cuneatus* body and foot, as shown in Table 2 was analyzed with the help of HPLC. Quantitative variation has been observed in the presence of amino acids in between two organs studied, whereas, Glycine is the major amino acid in both the extracted collagen. The imino acid contents (hydroxyproline and proline) of body and foot ASCs were 11.8 and 12.6%. In general, mammalian collagen contains more percentage of imino acids (Giraud Guille et al. 2000), but *D. cuneatus* ASCs had lower imino acid contents than mammalian collagens, which was in accordance with the report from Jongjareonrak et al. (2005). The hydroxyproline content was about 5.4 and 6.2% in both body parts. It is very important constituent in collagen because the hydroxylated proline plays an important role in stabilizing the triple helical structure (Ramachandran 1988). From the result, hydroxylation of proline in collagen from foot was slightly higher than that in collagen body, which suggests that collagen from foot, may have a slightly more complex structure than that from body as shown by the higher degree of hydroxylation (Kittiphattanabawon et al. 2005). Since tryptophan did not exist in the collagens from body and foot, also the contents of phenylalanine was very low, all ASCs do not have the absorption peak at 280 nm, but at 223 or 224 nm, which is accordance with results from Li et

al. (2004). Therefore, the collagen helices of *Donax cuneatus* might be less stable than those of mammalian, due to the lower imino acid content.

Conclusion

Acid soluble skin collagen was extracted from body and foot of *Donax cuneatus* and characterized using SDS PAGE, UV and FTIR spectrometry. It showed that both are having type I collagen. Results of HPLC proved the presence of both essential and non essential amino acids. Therefore, the present study has given the possibility to use the two body parts (body and foot) of wedge clam as an alternative source for porcine and cattle collagen, adds more value on this wedge clam *D. cuneatus* through the possibility of utilizing it in industries.

Table 2 Amino acid content of collagen from body and foot of *Donax cuneatus*

Amino acids	Body (%)	Foot (%)
Hydroxyproline	5.4	6.2
Aspartic acid	1.1	6.6
Threonine ^a	7.1	5.2
Serine	5.2	5.6
Glutamic acid	15.7	15.3
Proline	6.4	6.4
Glycine	20.5	19.2
Alanine	3.8	2.4
Valine ^a	4.7	6.2
Methionine ^a	1.5	0.9
Isoleucine ^a	2.7	2.6
Leucine ^a	5.8	4.7
Tyrosine	4.9	3.6
Phenylalanine ^a	1.1	0.7
Lysine ^a	4.0	2.2
Histidine	1.0	3.1
Arginine	9.1	9.1
Total	100	100
Imino acids	11.8	12.6

^a Essential amino acids

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