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Biochemical composition of the Australasian sea cucumber, *Australostichopus mollis*, from a nutritional point of view

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Abstract

Background: Sea cucumbers are highly prized seafood in Asia, where the demand for them has increased dramatically in recent years. However, nutritional information is only available for only a few of the commercially traded species.

Methods: In this study, the biochemical composition of the sea cucumber *Australostichopus mollis* was evaluated in terms of the major functional components: collagen, amino acid and fatty acid content using standard analytical procedures.

Results: The collagen, mostly type I, formed a homogeneously clustered network constituting 1.4% of the wet weight of adult *A. mollis* which is lower than for some other species of sea cucumber. The collagen consisted of α1 and α2 chains (around 116 kDa), α chain dimers, β chains (around 212 kDa), and small amounts of γ components. The most abundant amino acids were glycine, alanine, threonine, serine and proline (lysine/arginine ratio of 0.1). Threonine was the most abundant essential amino acid, followed by methionine and valine, while glycine was the dominant non-essential amino acid. The sea cucumbers had a low lipid content (0.2 and 0.1% of wet weight for the viscera and body wall, respectively) which is below the range reported for most other species of sea cucumber. The lipid contained high levels of PUFA (54%) compared to MUFA (23%) and SFA (24%). The dominant PUFA was arachidonic acid in both the body wall and viscera, followed by eicosapentaenoic acid.

Conclusions: Overall, the Australasian sea cucumber has strong potential as a functional food due to its high levels of PUFA and essential amino acids, comparing favourably with the most commercially valuable sea cucumbers.

Keywords: *Australostichopus mollis*, Holothurian, Beche-de-mer, Collagen, Amino acids, Fatty acids

Background

Sea cucumbers are marine invertebrates that have been consumed for centuries, especially in Asia, due to their nutritional and perceived health-giving properties [1]. Currently there are over 66 different species of sea cucumbers being harvested and widely traded in both tropical and temperate regions of the world [2]. The current high global demand for sea cucumbers has led to an overexploitation of many wild populations and the opening of new fishing grounds for new species in countries with no prior history of sea cucumber consumption or harvesting [2]. This includes the Australasian sea cucumber, *Australostichopus mollis*, for which commercial fisheries in New Zealand have rapidly developed in the last decade with a total allowable commercial catch of 35 tonnes [3]. The introduction of this species to Asian markets has met with strong initial demand and interest in the nutritional qualities of the species.

A. mollis is found all around New Zealand as well as on much of the southern coasts of Australia, inhabiting a wide range of habitats and depths where it is able to exploit different food sources [3]. This species can reach

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market values around US$275 kg^{-1} dry weight in Asia; although, the final price will depend on other factors such as the size and processing method [4]. However, additional value could be obtained for this species through the extraction and marketing of natural bioactive compounds which can be readily available in sea cucumbers [5]. So far, studies on the biochemical composition have focused on the pharmaceutical properties of molecules extracted from this species, such as triterpene glycosides [6–8]. Conversely, no studies have been carried out to characterise this species from a nutritional point of view for human consumption.

Sea cucumbers are generally regarded as a premium seafood since they usually have a high protein to lipid ratio and contain high levels of beneficial polyunsaturated fatty acids (PUFA), collagens, vitamins (e.g. A, B1, B2 and B3) and minerals (e.g. calcium, magnesium, iron and zinc) [5, 9–13]. However, it has been shown that the nutritional characteristics of sea cucumbers can vary greatly from species to species, and even within a species, factors such as distribution, food and processing method can also alter the nutritional value of the product [9, 11–14]. Lipid, fatty acids, collagen and amino acid composition have most frequently been associated with the functional food properties of sea cucumbers, and consequently, they have been a focus of analytical attention [9, 11, 15]. Therefore, the objective of this study was to determine the major biochemical components with nutritional value (i.e. collagen, amino acids and fatty acids) in the Australasian sea cucumber.

Methods

Sea cucumber collection and tissue preparation

Adult sea cucumbers (*A. mollis* of mean wet weight of 98 g and a mean length of 140 mm) used in the analyses were collected by scuba divers during June 2009 from a coastal site where they are abundant in north-eastern New Zealand (36° 19′ S, 174° 47′ E). The taxonomic identity of the sea cucumbers was confirmed independently by two sea cucumber experts with knowledge of this species. The live sea cucumbers were transported in seawater to the laboratory for analyses, thereby reducing the stress to the animals [16]. Once in the laboratory, 40 sea cucumbers (i.e. four sea cucumbers for each essay performed) were selected and weighed according to the procedure described by Sewell [17]. Then, the sea cucumbers were gutted using a surgical blade to separate the viscera (i.e. digestive tract, respiratory tree and gonads) from the body wall. Then, all tissue samples were carefully rinsed with water, blotted dry and transferred to a −80 °C freezer (Thermo Fisher Scientific, Forma, Waltham, MA, USA) for further analysis. All these procedures were carried out at 4 °C to reduce the enzymatic activities that could potentially autolyse the sea cucumber's tissues.

Moisture content

A small piece (5 × 5 cm) of sea cucumber body wall sample was mounted on a glass petri dish, and then covered with aluminium foil before placing in a vacuum oven (D-63450, Hanau Laboratory Products GmbH, Kendro, Germany) set at 70 °C for 12 h to provide for the rapid removal of moisture from the tissues [18]. The moisture content was calculated as percentage of weight loss after drying.

Collagen analyses

Extraction of crude collagen fibrils from the body wall

Collagen was extracted from samples of the body wall using a method based on Trotter et al. [19] with a slight modification by Cui et al. [10]. Samples of the frozen body wall (around 90 g wet weight) were thawed, washed and cut into small pieces. Then the small pieces were stirred in Milli-Q water (EMD Millipore Corporation, Bedford, MA, USA) twice at 300 rpm, for 30 and 60 min. After the second extraction in water, the liquid was replaced by the disaggregating solution (i.e. 4 mM ethylene-diamine-tetra-acetic (EDTA), 0.1 M Tris-HCl, pH 8.0) and left stirring overnight. Then, the samples were stirred slowly in Milli-Q water for 2 days. After centrifugation (Sorvall Evolution RC Centrifuge, Rotor SLA-3000, Newton, CT, USA) at 9000×*g* for 5 min, the supernatant was collected and the precipitate was stirred again with Milli-Q water overnight and centrifuged to recover a second supernatant. The supernatants obtained were combined, and then centrifuged at 10,000×*g* for 30 min to obtain a collagen precipitate, which was lyophilised in a freeze-dryer (Freezemobile 35ES, VirTis Inc., NY, USA). The freeze-dried crude collagen was further purified with 0.1 M NaOH for 3 days. The mixture was centrifuged at 15,000×*g* for 20 min and the precipitate of collagen fibril was washed three times with Milli-Q water and then lyophilised until all moisture was removed. The percentage of collagen present in the body wall was determined gravimetrically, by accurately weighing the freeze-dried collagen and then flushing with nitrogen and storing at −80 °C for further analysis (i.e. no more than a month later).

Isolation of pepsin-soluble collagen

The method for the isolation of pepsin-soluble collagen was derived from Nagai et al. [20] and Jongjareonrak et al. [21]. Purified collagen previously extracted from the body wall of the sea cucumber (around 100 mg) was weighed and then rehydrated with Milli-Q water. The rehydrate was then suspended in 50 ml of 0.5 M acetic acid and subjected to hydrolysis with porcine pepsin (EC 3.4.23.1., 56 units/mg solid, Sigma-Aldrich Co., St. Louis, MO, USA) at a pepsin/collagen ratio of 30/100 (*w*/*w*) for 2 days at 4 °C with gentle stirring (100 rpm). The viscous

solution was centrifuged at 20,000×*g* for 1 h at 4 °C, and the supernatant was recovered. To terminate the pepsin reaction, the supernatant was dialysed (Dialysis tubing membrane 12,000–14,000 Da, Visking Tubing, Medicell Membranes Ltd, London, UK) against 2 l of 0.02 M sodium phosphate buffer (pH 7.2) for 24 h at 4 °C with a change of solution every 4 h. The resulting dialysate was then centrifuged at 20,000×*g* for 1 h. The precipitate obtained was dissolved in 10 ml of 0.5 M acetic acid and centrifuged at 20,000×*g* for 1 h at 4 °C after addition of NaCl to a final concentration of 2.6 M. The resultant precipitate was collected and re-dissolved in 5 ml of 0.5 M acetic acid. This solution was dialysed with distilled water for 24 h at 4 °C with a change of solution every 4 h before lyophilizing. The total pepsin-soluble collagen (PSC) of the sample was evaluated gravimetrically, by weighing the freeze-dried matter obtained and then flushed with nitrogen and stored at −80 °C for later analysis (i.e. no more than a month later).

Scanning electron microscopy

The samples of extracted collagen were prepared as described previously by Yunoki et al. [22]. Small pieces (2 × 2 mm) of freeze-dried collagen and PSC, together with calf skin type I collagen, were mounted on scanning electron microscopy (SEM) stubs and coated with a thin layer of Pt. The coated samples were then visualised at a voltage of 5 kV with a SEM (Philips XL30S FEG, Eindhoven, The Netherlands), and images of the microstructure of each sample were obtained. Calf skin type I collagen (Sigma-Aldrich, Co., St. Louis, MO, USA) was used as a comparative standard for the identification of sea cucumber collagen.

Electrophoretic analysis

Protein patterns of collagen samples (both calf skin collagen and PSC samples) were analysed using gel electrophoresis a common method used for separation of collagen subunits [23]. The samples were dissolved in 0.1 M sodium phosphate buffer (pH 7.2) at a concentration of 1 mg ml^{-1}. The samples were prepared by mixing with a buffer (NuPAGE LDS) (Life Technologies Corporation, Carlsbad, CA, USA) and either β- mercaptoethanol or Milli-Q water for reduced and non-reduced samples. Afterwards, the samples were incubated at 70 °C at 300 rpm in a Thermomixer (Thermomixer compact, Eppendorf Instrumente GmbH, Hamburg, Germany) for 10 min. After incubation of the samples, together with a high molecular weight, markers from 53 to 212 kDa (GE Healthcare, Aylesbury, UK) were loaded onto the gel wells covered with buffer for the electrophoresis. The electrophoresis was carried out under a constant voltage of 200 V and a current of 111 mA for 1 h. After electrophoresis, the gel was stained with a solution prepared by dissolving 0.1% Coomassie brilliant blue in 40% ethanol and 10% acetic acid for 24 h, and then destained with a solution prepared using 10% ethanol and 7.5% acetic acid in Milli-Q water on a rotary shaker (RS-36, Remi Elektrotechnik Ltd, Mumbai, India). The images of protein sequence patterns were taken using a photo scanner (Scanjet 4370 digital Flatbed Scanner, Hewlett Packard Enterprise, Palo Alto, CA, USA).

Peptide mapping

Peptide mapping was conducted to determine the subunit composition of *A. mollis* collagen using modified methods of Lim et al. [24]. The lyophilized collagen samples were re-dissolved in 50 mM of NH_4HCO_3 at a concentration of 5 mg ml^{-1}. Dithiothreitol (DTT) was added to a final concentration of 10 mM, and the samples were heated at 56 °C for 1 h to reduce disulphide bonds. Samples were cooled, and then iodoacetamide was added to a final concentration of 50 mM to alkylate any cystine residues. After incubation in the dark for 1 h, further DTT equivalent to 10 mM was added to quench the residual iodoacetamide. Sequencing grade trypsin was added (Promega Corporation, Madison, WI, USA), and the samples were incubated at 37 °C overnight. Prior to liquid chromatography-mass spectrometry (LC-MS), tryptic digests were acidified to pH 2 using formic acid and then desalted on 10 mg Oasis HLB SPE cartridges (Waters Corporation, Milford, MA, USA) and eluted with 60% acetonitrile. The eluted material was concentrated in a vacuum centrifuge (Speed Vac-ISS110P1-115, Thermo Fisher Scientific) and then diluted fivefold with 0.1% formic acid, and 10 μl of this final sample was used for LC-MS analysis.

The HPLC system consisted of a PepMap C18 trap column (0.3 × 5 mm) (LC Packing) and a Zorbax 300SB C18 (0.3 × 100 mm) (Agilent Technologies, Santa Clara, CA, USA). Peptides were separated using a gradient of 0.1% (*v*/*v*) formic acid in Milli-Q water and 0.1% (*v*/*v*) formic acid in acetonitrile. The column eluate was directed into the ion source of a QSTAR XL hybrid quadrupole-time of flight (TOF) tandem mass spectrometry (Applied Biosystems, Foster City, CA, USA), after which a TOF mass spectrometry scan was performed from 300 to 1600 m/z, followed by the fragmentation of the three most abundant multiple charged species. The tandem mass spectrometry data were then searched against the metazoa subset of NCBI's protein sequence database as of November 2007 using the Mascot (v2.0.05) search engine. As the only protein hits of note were trypsin and keratin, the most promising MS/MS spectra were manually interpreted. Full or partial peptide sequences thus obtained were BLASTed against the metazoa subset of NCBI to determine any homology to known sequences. The manual determination de novo was carried out when the lack of automated matching occurred.

Ultraviolet spectra

The UV spectra were carried out to compare PSC of *A. mollis* with calf skin collagen to identify the type of collagens present. The samples were prepared by dissolving PSC and calf skin collagen with 0.5 M acetic acid. Each solution was then placed into a quartz cell with a path length of 1 cm. The UV absorption spectra were recorded using a spectrophotometer (UV-1700, Shimadzu Corporation, Kyoto, Japan) at wavelengths from 220 to 400 nm with an interval of 0.2 nm.

Differential scanning calorimetry (DSC)

DSC was done to determine the thermal stability of collagen of *A. mollis*. Collagen samples were prepared as described by Rochdi et al. [25]. The lyophilised collagen samples were rehydrated in 0.5 M acetic acid solution and allowed to stand for 2 days at 4 °C. The thermal transition of collagen was measured using a calibrated differential scanning calorimeter (Q1000, TA Instruments, New Castle, DE, USA) which measured differential heat capacity between a pan containing the sample and an empty reference pan. The rehydrated sample (5 mg) was weighed into aluminium pans, and then sealed tightly. The samples were scanned over the range of 10–80 °C at a heating rate of 2 °C min^{-1}. Liquid nitrogen was used as a cooling medium, and the system was equilibrated at 10 °C for 5 min prior to the scan. DSC transition curves of calf skin type I collagen were done as controls in the same manner and were compared to the samples. TA Instruments Universal Analysis Software (TA Instruments) was used to analyse the temperature data variation with different heat flows.

Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra were carried out to compare the molecular groups of PSC of *A. mollis* and calf skin collagen. PSC samples and calf skin collagen were analysed by a FTIR spectrometer (Spectrum 400, Perkin Elmer, Waltham, MA, USA). The spectra were recorded over the transmission range of 4000–500 cm^{-1} with automatic signal gain collected in 32 scans at a resolution of 4 cm^{-1}. A blank background spectrum was recorded at 25 °C as the reference. Analysis of spectral data (i.e. spectrogram peaks and absorbance) was carried out using Spectrum 10 data collection software.

Amino acid analysis of body wall and PSC

Hydrolysis of protein

Approximately 10 mg of either body wall or PSC sample was weighed and transferred into a 10-ml tube (Teflon-lined screw capped Kimax) (Thomas Scientific, Swedesboro, NJ, USA), together with L-norleucine internal standard (Sigma-Aldrich, Co.), and then, 2 ml of 6 M HCl was added and the tube was heated at 110 °C for 24 h in a heat block (QBT2, Grant Instruments Ltd, Shepreth, Cambridgeshire, UK) for hydrolysis. Then, the hydrolysates were cleaned-up to remove residual lipid from the sample prior to amino acid analysis [26]. The amino acid composition of viscera was not analysed due to a focus on the tissues of major biomass contribution.

Derivatization of amino acids

The cleaned hydrolysates were derivatized as a prerequisite for quantifying amino acids [27]. Aliquots of hydrolysate (100 μl) were dried under vacuum at room temperature, and 100 μl of 0.1 M HCl was added. Derivatisation was initiated by adding a drop of 50 μl of triethylamine (TEA) (Sigma-Aldrich Co.) reagent (two volumes of ethanol, two volumes of Milli-Q water and one volume of TEA) and 50 μl of derivatisation reagent (seven volumes of ethanol, one volume of Milli-Q water and one volume of TEA and one volume of phenylisothiocyanate), after which the solution was Vortexed (MS1 Minishaker, IKA Works (Asia) Sdn Bhd., Malaysia) and was allowed to stand at room temperature for 20 min to obtain the derivatised amino acid. Then, a few drops of n-hexane were added, and the solution was separated into two layers. Finally, 50 μl of the bottom layer was collected in a HPLC vial (Sigma-Aldrich Co.). The vial was filled up to 500 μl using sample diluent (5 volumes of acetonitrile to 95 volumes of 5 mM sodium phosphate buffer at pH 7.4), and then sealed tightly. Once the sample was prepared, the HPLC analysis was carried out immediately.

Analysis of amino acids composition

A Hewlett Packard 1100 Series HPLC system (Hewlett Packard Enterprise) was used for the separation of individual derivatised amino acids. The column used was a reserved phase Pico-Tag column (3.9 × 150 mm) (Waters Corporation) which was maintained at 38 °C in the column heater. The injection volume was 2 μl. The detector was set up at a fixed wavelength (254 nm), and the derivatised amino acids were separated using a gradient of two solvents; the first one was 0.14 M sodium acetate containing 0.5 ml l^{-1} TEA and titrated to pH 6.4 with glacial acetic acid. The second solvent was 60% acetonitrile. After this, a washing step was programmed to clean the second solvent completely to eliminate any residual sample components from the column. The derivatised amino acids were identified and quantified by comparing their retention times with those of amino acids present in standard calibration curves [27]. The calibration curves were created using an amino acid standard H (Thermo Scientific) containing 2.5 μmol ml^{-1} for each amino acid in 0.1 M HCl (except cystine, 1.25 μmol ml^{-1}). This standard did not contain the secondary amino acids, hydroxylysine and hydroxyproline, as it was designed to

determine the suite of amino acids of primary importance in human nutrition. Different volumes of the standard were used for the calibration curves to have different concentrations from 0 to 2.5 μmol ml^{-1} for each amino acid (except cystine, in which the concentrations was half of others). The calibration curve for each amino acid was constructed by plotting the mean peak area for each concentration. Then, the content of each amino acid in a sample was determined by interpolation of the respective peak area of the amino acid. Only in the case of hydroxyproline, information from the literature was used to identify its presence in the samples by the position of its peak in the HPLC chromatogram [26].

Lipid and fatty acids analyses

Lipid extraction

Frozen samples of both sea cucumber body wall and viscera were thawed at 4 °C in a refrigerator. Then, the samples were homogenised at 16,000 rpm for 10 min using an Ultra-Turrax homogeniser (T-25, IKA-Werke GmbH & Co., Staufen, Germany) prior to lipid extraction. The lipid extraction method applied in this study was a modified Bligh and Dyer extraction [28]. Approximately 100 g of the homogenate of either body wall or viscera was mixed with 100 ml cyclohexane and 80 ml isopropanol, and blended for 5 min using an Ultra-Turrax at 11,000 rpm. Milli-Q water (110 ml) was added into the sample afterwards and blended for another 2 min. The phases were separated by centrifugation at 750×*g* for 5 min, and the top layer was transferred into a flask. A 50 ml of a mixture of cyclohexane/isopropanol was added into the residue, and blended for 2 min for the second extraction. After centrifuging, the supernatant was collected and combined with the first extraction in the flask. The combined supernatant was evaporated using a rotary evaporator at 40 °C to remove the solvent. The flask was then rinsed twice with 1 ml of chloroform/methanol. The lipid extract was transferred into a small glass vial, and the vial was then placed in a water bath at 80 °C for 20 min to evaporate the solvent. The vial was put in a forced air convection oven for 90 min at 103 °C to evaporate the solvent. The weight of extracted lipid was recorded and then flushed with nitrogen and stored at −80 °C for later analysis (i.e. no more than a month later). The total lipids of the sample were quantified gravimetrically.

Fatty acid composition

Determination of the fatty acid composition was done according to the method reported by Hartman and Lago [29]. Approximately 20 mg of lipid extracted from sea cucumbers using the method described previously was weighed and transferred into a 20 ml Kimax tube. Then, 300 μl of 4 mg ml^{-1} internal standards (tridecanoic acid, C:13 and tricosanoic acid, C:23. Sigma-Aldrich Co.) was added, followed by 0.5 ml of a saponifying agent. The solution was heated in a water bath at 65 °C for 10 min, and then, 3 ml of methylation agent (i.e. prepared by adding NH_4Cl to methanol then adding concentrated sulphuric acid, heating the mix in a water bath) was added, and heated at 65 °C for another 5 min. After the mixture cooled down, 10 ml of hexane was added, and shaken well for 2 min. After the two layers were separated, the hexane layer was transferred to another Kimax tube and dried over calcinated Na_2SO_4. Approximately 1 ml of the hexane layer with the fatty acid methyl esters (i.e. FAME) was transferred into a gas chromatography vial and sealed under nitrogen for further analysis. Gas chromatography (CG), using a Hewlett Packard 5890 Series II with a DB-225 fused silica capillary column (50/50 of cyanopropylmethyl/methylphenyl silicone phase, 0.25 mm in diameter, 0.25 μm in film thickness, 30 m in length) was used to separate FAME, using helium as carrier gas (33 ml min^{-1} flow rate). The GC was equipped with a FID and a capillary inlet with electronic pressure control. The sample was injected into the GC using a Hewlett Packard Series 6890 auto-injector (Hewlett Packard Enterprise). The GC oven was initiated at 40 °C for 3 min followed by ramping to 220 °C, reached by an increase of 4 °C min^{-1}, where it was held for 7 min. The total running time was 55 min for each sample. The pressure was run at an initial column head pressure of 10 psi in a constant flow mode, which is equivalent to 1.6 ml min^{-1} column flow. The flow rate of the split at injection was 33 ml min^{-1}. The FAME present in the sea cucumbers were identified by comparing their retention times with those of the FAME contained in the standards (by mixing a commercially available 37-FAME standard with docosapentaenoic acid methyl ester, cis-vaccenic acid methyl ester, and octadecatetraenoic acid methyl ester. Sigma-Aldrich Co.) used to build the calibration curves. Calibration curves were obtained for each FAME when diluting the standard at different known concentrations. When the FAME in the samples could not be related to those present in the standard, they were identified by using information available in the literature. Each fatty acid was quantified by interpolating the peak area obtained for its respective calibration curve and then calculated as a relative percentage of the total mass of fatty acids in the lipid sample. Total proportions of polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) were calculated by adding mean values for the relative proportions of the fatty acids within each grouping.

Statistical analyses

For all analyses, four replicate individual sea cucumbers were used. The data were reported as mean value ±

standard deviation for each group. Pairs of mean values for biochemical constituents were compared using t tests at significance level of 5%. The analysis was done using SPSS 17 software (SPSS Inc., Chicago, IL. USA).

Results

Total moisture and collagen content

The body wall of *A. mollis* was composed mostly of water with 93% of its wet weight, while collagen only constituted 1.4% of their wet weight.

Collagen characteristics and structure

The SEM observations of *A. mollis* collagens demonstrated well-developed fibril networks with thin, relatively uniform and densely interwoven fibrils of collagen consistent with a homogeneously clustered network (Fig. 1a). The PSC sponges extracted from the body wall of the sea cucumber were loose and porous, compared with the non-pepsin-digested collagen (Fig. 1b).

The electrophoresis results showed that the collagen in *A. mollis* consisted of α1 and α2 chains (around 116 kDa), α chains dimer, β chains (around 212 kDa) and small amounts of γ components (Fig. 2). The electrophoresis patterns were similar to those of calf skin collagen. Generally, there were no differences in the patterns of PSCs observed with or without β-ME, which indicated that both collagen fractions contained no disulfate bonds (Fig. 2). Peptide mapping showed high homology to various α type collagens with the major sequences identified as α collagens, α1 type I, and α2 type I and some sequences such as α4 type IV, α type V, α type XIX, as well as procollagen. $\alpha1(I)_2\alpha2(I)$ heterotrimers were also identified in the *A. mollis* body wall collagen.

The maximum UV absorbance of *A. mollis* collagen was at 234 nm which was similar to calf skin type I collagen at 232.4 nm (Fig. 3a). The collagen of *A. mollis* has a significantly lower mean ($P < 0.05$) thermal denaturation temperature (i.e. 46.7 °C) than calf skin collagen (Fig. 3b).

From FTIR spectra which was used to compare the molecular groups comprising PSC of *A. mollis*, amide A, amide B, amide I, amide II, amide III and four other bands without common names were observed (Fig. 3c). The triple helical structures of calf skin collagen and PSC were confirmed from the amide III band, and the FTIR spectrograms peaks from 1200 to 1500 cm^{-1} indicated a high level of glycine and proline residues in the collagen.

Amino acid composition

In *A. mollis*, of the essential amino acids, threonine was the most abundant in the sea cucumber body wall and pepsin-soluble collagen, followed by methionine and valine (Table 1). While for the non-essential amino acids, glycine was the most abundant and other amino acids, such as alanine, proline, serine and glutamic acid, were also present at reasonably high levels (Table 1). Only low levels of phenylalanine, cystine and lysine were detected in the sea cucumber body wall and pepsin-soluble collagen, while hydroxylysine was not detected (Table 1). There were differences in the mean amounts of amino acids found among the PSC and body wall of sea cucumbers ($P < 0.05$), and the profile of amino acids from the two different components of the sea cucumbers also appeared to be different from the amino acid profile of calf skin collagen (Table 1). However, the non-essential amino acids in the body wall of the sea cucumbers were similar to those of the calf skin collagen, but lower than those of the PSC samples ($P < 0.05$) (Table 1). Whereas for the essential amino acids, the levels found in both the body wall and in the PSC samples were generally higher than those previously found in calf skin collagen (Table 1).

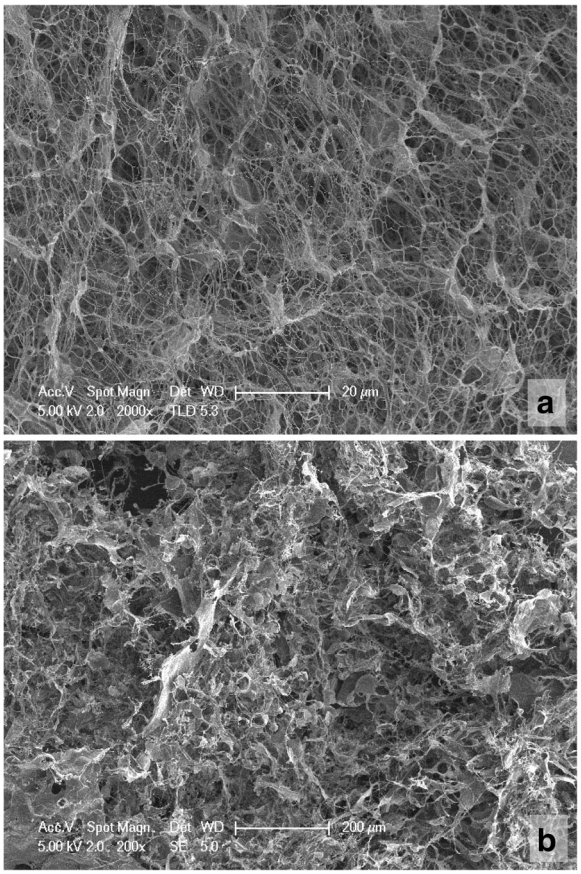

Fig. 1 SEM image of the collagen (**a**) and pepsin-soluble collagen (PSC) (**b**) extracted from the body wall of the Australasian sea cucumber (*A. mollis*). Scale bar for **a** 20 μm and for **b** 200 μm

Lipid and fatty acid composition

The body wall and viscera of *A. mollis* contained a total amount of lipid corresponding to 0.2 and 0.1% of wet weight, respectively. The lipid content in the viscera was significantly higher than in the body wall ($P < 0.05$).

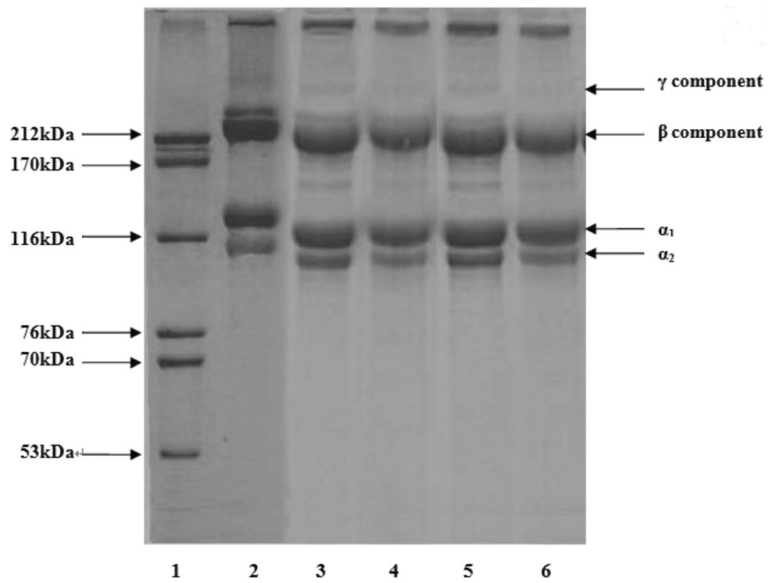

Fig. 2 Electrophoresis patterns of pepsin-soluble collagen (PSC) extracted from the body wall of the Australasian sea cucumber (*A. mollis*) under reducing and non-reducing conditions. *1* High molecular weight markers. *2* Calf skin collagen. *3–4* PSC (non-reducing). *5–6* PSC (reducing)

The fatty acids present in the body wall and viscera of *A. mollis* showed a predominance of polyunsaturated fatty acids (PUFA), while saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) contents were of similar extent (Table 2). There were no significant differences in the mean SFA, MUFA and PUFA between the body wall and viscera ($P > 0.05$) (Table 2). When comparing the proportions of individual fatty acids, there were no significant differences between the body wall and viscera ($P > 0.05$). However, the total n-3 fatty acid content was significantly higher in the viscera than in the body wall ($P < 0.05$) (Table 2).

The dominant PUFA was arachidonic acid, C20:4 (n-6) (AA) in both the body wall and viscera, and the second most abundant was eicosapentaenoic acid, C20:5 (n-3) (EPA) (Table 2). The content of docosahexaenoic acid, C22:6 (n-3) (DHA), was much lower than AA and EPA but similar to stearidonic acid, C18:4 (n-3) (STD) (Table 2). In terms of MUFA in *A. mollis*, eicosenoic acid C20:1 (n-9) was the most abundant MUFA, followed by palmitoleic acid C16:1 (n-7). Other MUFA, such as cis-vaccenic acid C18:1 (n-7) and its isomer *cis*-C18:1 (n-9) and C24:1 (n-9), were also present in reasonably high levels (Table 2). The main SFA in *A. mollis* in decreasing order of abundance were palmitic acid C16:0, and stearic acid C18:0 and C20:0 (Table 2).

Discussion

Collagen in sea cucumbers

Collagen is an important component for determining the palatability of sea cucumbers, especially the presence of high molecular weight cross-linked molecules which increase with age or starved animals [30]. Therefore, well-fed young animals coming from aquaculture practices could potentially guarantee better quality product than the sea cucumbers landed by fisheries. In the case of *A. mollis*, as in most sea cucumbers, the major edible component is the body wall which is mainly constituted by water and collagen. Collagen can comprise as much as 30% of the total protein in the body wall of sea cucumbers [10]. Another temperate species, *Apostichopus japonicus*, has been reported to contain more than ten times more collagen than measured in *A. mollis* [31]. The collagen fibres in sea cucumbers as observed in *A. mollis* are also responsible for the mechanical properties of the dermis and for the maintenance of the tissue integrity [10, 13, 20, 21, 32]. The collagen structure in *A. mollis*, dominated by type I collagen with α1 and α2 subunits, is similar to what has been reported for *Schizosaccharomyces japonicus* and *Cucumaria frondosa* and other echinoderms [10, 19, 32]. The lower thermal denaturation temperature in *A. mollis* (i.e. 46.7 °C) is probably due to the lower levels of hydroxyproline and proline in their collagen when compared to the calf skin [10, 27].

Amino acids in sea cucumbers

The amino acid composition of sea cucumbers changes from species to species and even within the same species depending on its distribution [10–13, 33]. The body wall and PSCs of *A. mollis* were rich in glycine, alanine, threonine, serine and proline, at levels similar to what has been found in China for the highly valued *A. japonicus* which is sold widely in Asian markets [10]. However,

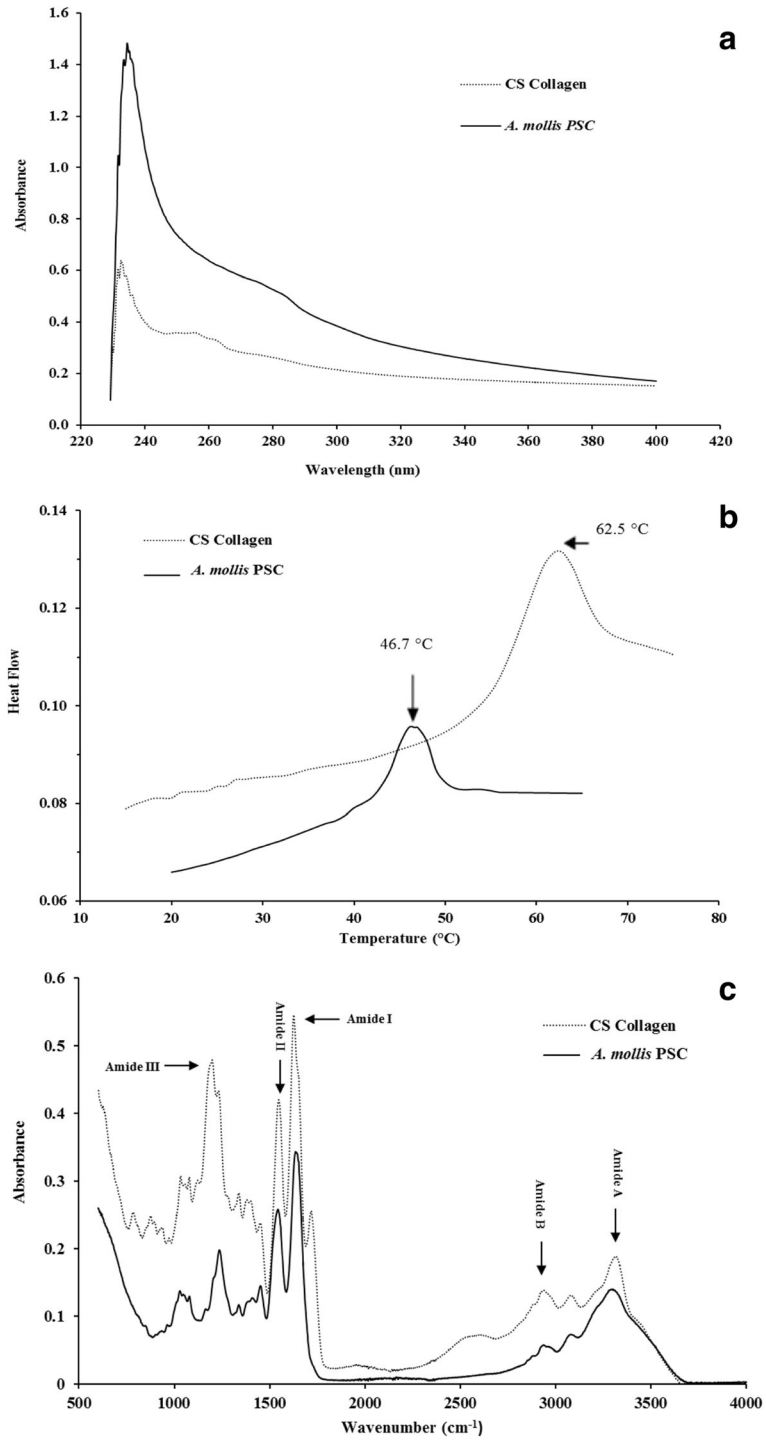

Fig. 3 UV spectra (**a**), DSC thermograms (**b**), and FTIR spectra (**c**) of pepsin-soluble collagen (PSC) extracted from the body wall of the Australasian sea cucumber (*A. mollis*) and from calf skin (CS) type I collagen

analyses of *A. japonicus* from Korea found that the most abundant amino acids in its body wall in descending order were glutamic acid, aspartic acid, arginine and glycine, which could be due to habitat and feeding differences [11]. In another temperate species of sea cucumber, *C. frondosa*, the most abundant amino acids were glutamic acid, glycine, aspartic acid and alanine [13]. While the most abundant in tropical species of sea cucumber are glycine, glutamine, aspartic acid and alanine [12]. Overall, for most species of sea cucumbers,

Table 1 Amino acid composition of the Australasian sea cucumber (*A. mollis*) body wall and pepsin-soluble collagen (PSC)

Amino acids	*A. mollis*		Calf skin
	Body wall	PSC	
Essential	Residues/1000 residues		
Threonine	98.67 ± 0.55[a]	83.96 ± 0.37[b]	18
Valine	20.69 ± 0.41[a]	13.06 ± 0.05[b]	21
Methionine	59.13 ± 0.74[a]	62.25 ± 0.11[b]	6
Isoleucine	10.47 ± 0.09[a]	10.66 ± 0.02[a]	11
Leucine	19.13 ± 0.07[a]	13.46 ± 0.09[b]	23
Phenylalanine	5.32 ± 0.05[a]	6.51 ± 0.16[b]	3
Lysine	5.24 ± 0.12[a]	4.36 ± 0.02[a]	26
Total	218.63 ± 2.02[a]	197.82 ± 0.81[b]	108
Non-essential			
Aspartic acid	53.81 ± 0.76[a]	30.15 ± 0.03[b]	45
Glutamic acid	54.08 ± 0.42[a]	61.62 ± 0.07[b]	75
Serine	95.64 ± 0.09[a]	86.23 ± 0.41[b]	39
Glycine	264.85 ± 0.45[a]	286.19 ± 1.59[b]	330
Histidine	19.94 ± 0.12[a]	19.44 ± 0.27[a]	5
Arginine	41.19 ± 0.63[a]	46.03 ± 0.22[b]	50
Alanine	155.38 ± 0.47[a]	159.05 ± 0.34[a]	119
Proline	66.05 ± 0.12[a]	87.93 ± 1.75[b]	121
Tyrosine	24.66 ± 0.24[a]	26.57 ± 0.10[a]	3
Cystine	5.81 ± 0.075[a]	8.96 ± 0.02[b]	N/A
Total	781.38 ± 3.34[a]	802.14 ± 4.77[b]	787
Others			
Hydroxyproline	ND	ND	94
Hydroxylysine	ND	ND	7

Results from sea cucumber are mean values of four replicates ± standard deviation. Mean values for sea cucumber in each row with the same superscript are not significantly different ($P > 0.05$) from one another using a *t* test. Calf skin values provided as a comparison were obtained from Li et al. [43]
ND not determined

the most abundant levels of amino acids correspond to the non-essential amino acids, whereas only *A. mollis* has been found to have high levels of the essential amino acid, threonine. In *A. mollis*, the ratio of essential amino acids to non-essential amino acids is relatively small (i.e. 0.3) compared to tropical sea cucumber species with ratios between 0.4 and 0.6, and when compared with the temperate *C. frondosa* and *A. japonicus* with ratios ranging from 0.3 to 0.7 [11–13, 31]. Nonetheless, the high composition of essential amino acids in the collagen of *A. mollis* reflects the premium quality of protein from this species [13]. In addition *A. mollis* presented a very low lysine:arginine ratio (i.e. 0.11) compared to *A. japonicus* (i.e. 0.48), *C. frondosa* (0.96) and to other tropical sea cucumber species (0.13–0.39) [10–13]. This low lysine: arginine ratio suggests that *A. mollis* is an ideal

Table 2 Fatty acid composition expressed as a percentage of the total fatty acids of the Australasian sea cucumber (*A. mollis*) body wall and viscera

Fatty acid	Common name	Body wall	Viscera
SFA			
C12:0	Lauric acid	0.64 ± 0.09[a]	0.27 ± 0.05[b]
C14:0	Myristic acid	1.27 ± 0.13[a]	1.75 ± 0.13[a]
C16:0	Palmitic acid	8.64 ± 0.71[a]	7.72 ± 0.49[a]
C17:0	Heptadecanoic acid	0.89 ± 0.13[a]	0.85 ± 0.08[a]
C18:0	Stearic acid	4.77 ± 0.28[a]	3.49 ± 0.32[b]
C20:0	Arachidic acid	2.74 ± 0.26[a]	2.79 ± 0.28[a]
C21:0	Heneicosanoic acid	2.47 ± 0.18[a]	1.79 ± 0.15[b]
C22:0	Behenic acid	2.24 ± 0.19[a]	2.04 ± 0.14[a]
Total SFA		23.65 ± 1.94[a]	20.69 ± 1.61[a]
SFA (g kg^{-1})		0.24 ± 0.02	0.41 ± 0.04
MUFA			
C16:1 (n-7)	Palmitoleic acid	3.41 ± 0.28[a]	5.50 ± 0.75[a]
C17:1 (n-7)	*cis*-10-Heptadecenoic acid	0.29 ± 0.03[a]	0.22 ± 0.03[a]
C18:1 (n-7)	*cis*-Vaccenic acid	2.92 ± 0.22[a]	2.77 ± 0.20[a]
C18:1 (n-9) *cis*	Oleic acid	4.38 ± 0.19[a]	2.59 ± 0.09[b]
C20:1 (n-9)	Eicosenoic acid	8.34 ± 0.61[a]	7.74 ± 0.54[a]
C22:1 (n-9)	Erucic acid	0.61 ± 0.07[a]	0.73 ± 0.04[a]
C24:1 (n-9)	Nervonic acid	2.67 ± 0.21[a]	2.90 ± 0.40[a]
Total MUFA		22.61 ± 1.60[a]	22.44 ± 2.36[a]
MUFA (g kg^{-1})		0.23 ± 0.02	0.45 ± 0.04
PUFA			
C18:3 (n-3)	Linolenic acid	0.70 ± 0.01[a]	0.70 ± 0.04[a]
C18:4 (n-3)	Stearidonic acid (STD)	4.76 ± 0.14[a]	6.71 ± 0.20[b]
C20:3 (n-3)	Eicosatrienoic acid	1.23 ± 0.07[a]	1.44 ± 0.08[a]
C20:5 (n-3)	Eicosapentaenoic acid (EPA)	10.63 ± 0.66[a]	12.51 ± 0.21[b]
C22:5 (n-3)	Docosapentaenoic acid (DPA)	0.52 ± 0.04[a]	0.56 ± 0.05[a]
C22:6 (n-3)	Docosahexaenoic acid (DHA)	4.64 ± 0.315[a]	6.44 ± 0.23[b]
C18:2 (n-6)	Linoleic acid	0.67 ± 0.04[a]	0.62 ± 0.12[a]
C18:3 (n-6)	γ-Linolenic acid	1.15 ± 0.04[a]	0.55 ± 0.12[b]
C20:2 (n-6)	Eicosadienoic acid	1.19 ± 0.09[a]	1.27 ± 0.11[a]
C20:3 (n-6)	Dihomo-γ-linolenic acid	0.37 ± 0.02[a]	0.45 ± 0.06[a]
C20:4 (n-6)	Arachidonic acid (AA)	27.9 ± 0.39[a]	25.91 ± 1.31[a]
Total (n-3)		22.47 ± 1.23[a]	28.34 ± 0.79[b]
Total (n-6)		31.28 ± 0.58[a]	28.79 ± 1.72[a]
Total PUFA		53.75 ± 1.81[a]	57.13 ± 2.51[a]
PUFA (g kg^{-1})		0.54 ± 0.02	1.14 ± 0.05

Total SFA, MUFA and PUFA as a proportion of total fatty acids are presented as well as their quantities measured as gram per kilogram of wet body wall and viscera. Results are mean values of four replicates ± standard deviation. Values with the same superscript in each row are not significantly different ($P > 0.05$) from one another

diet for people with high cholesterol levels as it could help reduce its levels in the blood and therefore hypertension [18].

Lipid and fatty acids in sea cucumbers

The overall lipid content of *A. mollis* is lower than the reported values for both tropical and temperate sea cucumber species which are within the range of what has been observed in other species [13, 15, 34]. In addition, *A. mollis* having more lipids in the viscera than in the body wall supports the use of these internal organs as energy reserve structures for periods of food scarcity [35]. The viscera of sea cucumbers are considered delicacies that are consumed either dried or fermented in some Asian countries, but that are mostly wasted during processing of most species [36].

Overall, the fatty acid composition of *A. mollis* is very similar to what has been previously reported for other species of sea cucumbers in terms of relative proportions of SFA, MUFA and PUFA [12–15, 31, 34, 37]. When compared with other valuable seafood, the levels of PUFA in the body wall (54 mg/100 g wet), MUFA (23 mg/100 g wet) and SFA (24 mg/100 g wet) reported for *A. mollis* are below their levels [38]. Fatty acid levels reported for other commonly consumed seafood in the Australasian region such as oysters, shrimps, lobsters and most finfish can be over 100 mg/100 g wet weight depending on the species [38]. As mentioned before, the proportional fatty acid composition of *A. mollis* was characterised by high levels of PUFA, especially arachidonic acid. This fatty acid is the main PUFA in other sea cucumber species, especially in tropical species [12, 14, 15, 34]. Other PUFA, such as EPA and DHA, were also found at relatively high proportionate levels in *A. mollis*, which is consistent with many other sea cucumber species [34]. Although sea cucumbers can be consumed raw, most of them are processed post-harvest and commercialised in a dry format. Drying the sea cucumbers using traditional Asian processing methods involves repeated boiling and drying which can reduce the amount of PUFA to around 10% due to oxidation [4, 9, 12]. Alternative methods of processing sea cucumbers, such as freeze drying, have been developed to reduce the loss of bioactive components, such as PUFA, and to increase the unique health food qualities for which sea cucumbers are known [39]. It has been proven that PUFA are effective for the treatment and prevention of a number of diseases, which makes sea cucumbers an alternative source of these fatty acids that cannot be synthesised de novo by humans [40, 41]. The lipid composition does not only add to their nutritional and health-giving properties but also can influence the flavour and storage characteristics of products derived from these valuable animals [42].

Conclusions

The nutritional composition of *A. mollis* in terms of collagen, amino acids and fatty acids indicates that this species has a broadly similar composition to other sea cucumber species which are widely used as functional foods and in traditional medical practice to treat and prevent several illnesses. However, the overall lipid and collagen content of this species were generally lower than in other species of sea cucumber. The collagen composition was dominated by type I collagen with α1 and α2 subunits, similar to the temperate sea cucumber species, *A. japonicus* and *C. frondosa*, as well as other echinoderm species for which collagen composition has been examined. The essential amino acids threonine, methionine and valine were dominant in the sea cucumber body wall and in the pepsin-soluble collagen. The lipid of *A. mollis* contained a high proportion of PUFA versus MUFA and SFA compared to other sea cucumber species. The two dominant PUFA were arachidonic and eicosapentaenoic acid. Further research is required to determine how environmental factors, such as food availability and seawater temperature which could change throughout the distribution of this species, could affect the nutritional quality of *A. mollis*.

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Availability of data and materials

The datasets during and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FL, SYQ and AJ conceived the study and carried out the analyses, data interpretation and drafted the manuscript. LZ and AJ helped with the data interpretation and drafted, revised and finalised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethics approval

The research was compliant with New Zealand's Animal Welfare Act 1999. The collection of animals was carried out under Special Permit 453 pursuant to section 97(1) (a) (i) and (ii) of the Fisheries Act 1996, New Zealand.

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