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Preservation of the bioactive saponins of *Holothuria scabra* through the processing of trepang

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Abstract: Holothuroids produce highly diverse natural organic compounds which present interesting nutritional and pharmacological properties. This is why processed sea cucumbers, also called trepang, have a high commercial value and are consumed for food and traditional medicine in Asian communities. Among bioactive substances, saponins are secondary metabolites structurally known as triterpene glycosides. Holothuroid saponins possess a wide spectrum of pharmacological effects including hemolytic, cytostatic, antitumoral, antifungal and antibacterial activities. This study intends to analyse and to compare the saponins contained in the body wall of *Holothuria scabra*, in the water used to prepare those organisms into trepang, and finally in the trepang itself. Saponins were extracted and purified by chromatography and liquid-liquid extraction methods and were then analysed by mass spectrometry (MS and MS/MS). Results show that the body wall of *H. scabra* contains six major saponins, with a mean concentration of 1 g.kg^{-1} of tissue (wet weight). The same saponins were also found in trepang with roughly the same concentration but a different proportion. In cooking water, only some saponin fragments are detected indicating that the small amount of saponin which is extracted is presumably thermally hydrolysed.

Résumé : *Préservation des saponines bioactives d'*Holothuria scabra *au cours de la transformation en trépang.* Les holothuries produisent une grande diversité de composés organiques naturels présentant d'intéressantes propriétés nutritionnelles et pharmacologiques. C'est pourquoi, les concombres de mer sont transformés en produits finis, appelés trépangs, qui ont une grande valeur commerciale et sont consommés pour leurs propriétés alimentaires et médicinales par les communautés asiatiques. Parmi les substances bioactives, les saponines sont des métabolites secondaires appartenant à la classe structurelle des glycosides triterpéniques. Les saponines d'holothurie possèdent un large spectre d'effets pharmacologiques tels que des activités hémolytiques, cytostatiques, antitumorales, antifongiques et antibactériennes. Cette étude a pour but d'analyser et de comparer les saponines présentes dans le tégument d'*Holothuria scabra*, dans l'eau de cuisson du trépang, et finalement dans le trépang d'*H. scabra*. Les saponines ont été extraites et purifiées par chromatographie et extractions liquide-liquide, puis analysées par spectrométrie de masse (MS et MSMS). Les résultats montrent que le tégument de *H. scabra* contient six saponines principales avec une concentration moyenne d'1 gramme de saponine par kilogramme de tégument (poids frais). Les mêmes saponines ont été également détectées dans le trépang avec une concentration similaire mais dans des proportions différentes. Dans l'eau de cuisson, seuls certains fragments de saponines ont été détectés, ce qui indique que le peu de saponines ayant été extraites sont vraisemblablement hydrolysées thermiquement.

Keywords: Saponins • Holothuroids • Trepang • Mass spectrometry • Sandfish

Introduction

Holothuroids, or sea cucumbers, produce highly diverse natural organic compounds which present interesting nutritional and pharmacological properties (Bordbar et al., 2011). Among these bioactive substances, saponins were detected in many holothuroid species and are present in their body wall, internal organs and Cuvierian tubules (Caulier et al., 2011). Saponins are natural secondary metabolites first discovered in many higher plants (*e.g.* the soap tree *Quillaja saponaria*) (Li et al., 2006). Through the research for new pharmacologically active substances, saponins have also been isolated from different marine organisms such as holothuroids (Nigrelli, 1952; Yamanouchi, 1955), sea stars (Mackie & Turner, 1970) and sponges (Thompson et al., 1985). In ecology, saponins are deleterious for most organisms and probably function as a chemical defense to deter predation (Kalinin et al., 1996a ; Van Dyck et al., 2011).

Structurally, holothuroid saponins are triterpene glycosides composed of an aglycone (sapogenin) and a glycosidic part. The aglycone backbone is based on holostane-3β-ol and the carbohydrate chain encloses up to 6 sugars units, including xylose, glucose, 3-*O*-methylglucose and quinovose, and can be branched only once (Stonik & Elyakov, 1988). Most of the time, the oligosaccharide is attached at the C3 position of the sapogenin. The particular structure of saponins confers them amphiphilic and tensioactive properties which are at the origin of a wide range of pharmacological effects including hemolytic, antitumoral, anti-inflammatory, antifungal, anti-bacterial, antiviral, ichthyotoxic, cytostatic and antineoplastic activities (Kerr & Chen, 1995; Kalinin et al.,1996a & b; Prokofieva et al., 2003).

For centuries, sea cucumbers have been consumed by Asian communities as delicacy food and traditional Chinese medicine. Therefore, processed sea cucumbers, also called trepangs, present a high commercial value on Chinese markets (Conand, 2004). The Indian Ocean Trepang (IOT) is a sea cucumber farm industry based in the South West of Madagascar, Toliara, which produces and prepares trepangs from the most valuable local exploited sea cucumber species, the sandfish *Holothuria scabra* (Jaeger, 1833). IOT also exports final products to the international market. Trepang processing involves sea cucumber evisceration, salting, cooking for several hours in boiling water, and finally drying into trepang (Conand, 1979). An additional step to remove the abundant calcareous spicules present in the outer integument of this species consists of scraping the animal with a stone or bivalve shell, or using ground papaya leaves to enzymatically digest the outer dermis (Lavitra et al., 2008).

Some saponins of *H. scabra*, extracted from the body wall or the internal organs, have already been chemically characterized (Thanh et al., 2006; Dang et al., 2007; Hua et al., 2009). However, no study has ever highlighted the presence of saponins in the commercially available sandfish trepangs. The aim of this study is to compare the saponins contained in (a) the integument of a fresh *H. scabra* (Fig. 1A), (b) the trepang (Fig. 1B) and (c) the cooking water, in order to discover if saponins are preserved during trepang processing.

Materials and Methods

Three fresh individuals of *H. scabra*, three trepangs and three samples of 100 ml of cooking water were provided by the company Indian Ocean Trepang (IOT) based in Toliara, South-West of Madagascar. The fresh sea cucumbers were eviscerated and only their body wall was preserved in methanol 99.9%. Trepangs prepared by IOT were transported dried to the University of Mons, Belgium, whereas samples of cooking water were frozen and stored at -20°C during the transport. Saponin extraction, quantification and analyses were realised at the University of Mons.

Trepangs were first rehydrated for 1 week in distilled water to recover their original volume. Indeed, original

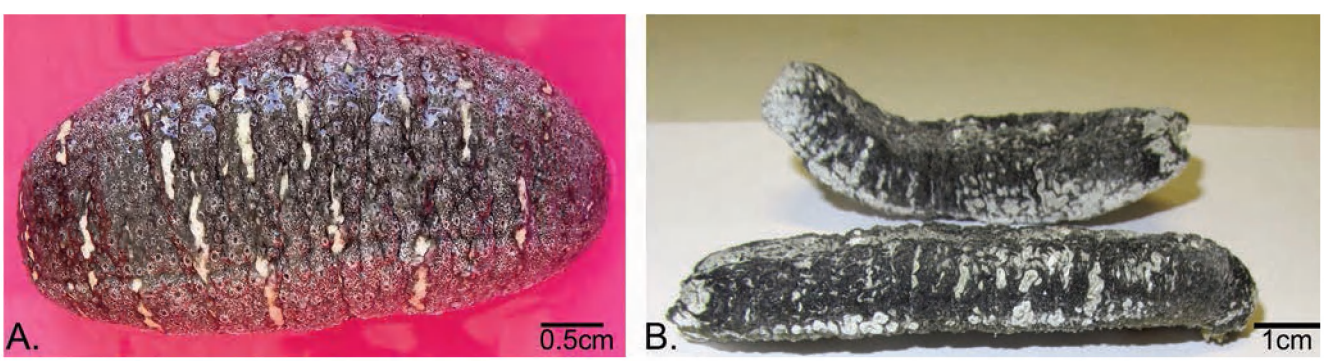


Figure 1: Sea cucumber *Holothuria scabra* before (**A**) and after (**B**) trepang processing.

weight of dried trepangs averaged 11.3 g in comparison to 64 g when fully rehydrated. The average weight of body walls was 57.5 g. Then, tissues of fresh holothuroids and trepangs were homogenized in methanol. After filtration, the methanolic extract was partitioned successively against n-hexane (v/v), dichloromethane (v/v) and chloroform (v/v). The methanolic extract was then evaporated under low pressure using a rotary evaporator (Laborota 4001 efficient, Heidolph) and dissolved in water in order to undergo chromatographic purification. The crude aqueous extract was placed on a column of Amberlite XAD-4 (Sigma-Aldrich, St Louis, MO, USA). Washing the column with water removed the inorganic salts and subsequent elution with methanol allowed to recover saponins. The methanolic phase was then evaporated and the dry extract was diluted in water in order to undergo a last partitioning against isobutanol (v/v). The butanolic fraction, containing the purified saponins, was evaporated to dryness and weighted. Saponin concentrations were then calculated by dividing the dry weight of the saponin extracts by the wet weight of the samples (rehydrated weight in the case of trepang). Non-parametric statistic Mann-Whitney test was used to compare the saponin concentration contained in the integument and in trepang. Cooking water samples were directly loaded on the chromatography column and then the same procedure was followed to prepare the sample for the mass spectrometry analysis.

For mass spectrometry analyses, saponins were then re-dissolved in acetonitrile. All analyzes were performed on a Water QToF Premier mass spectrometer using MALDI source in the positive ion mode. The MALDI source was a nitrogen laser, operating at 337nm with a maximum output of 500 mW delivered to the sample in 4 ns pulses at 20 Hz repeating rate. The matrix used was a mixture of di-hydro benzoic acid (25 mg) and dimethylaniline (5 µl) (DHB-DMA) in 250 µl of acetonitrile/water, 1/1. The matrix solution (1 µl) was spotted onto a stainless steel target and air-dried. Then, 1 µl of each saponin extracts was applied onto the spots of matrix crystals and air-dried. For the recording of the single-stage MALDI-MS spectra, the quadrupole (rf-only mode) was set to pass ions between m/z 50 and 1500, and all ions were transmitted into the pusher region of the time-of-flight (TOF) analyzer where they were mass-analyzed with a 1 s integration time. The proportions of the different congeners in the samples were estimated by comparing the relative intensities of the saponin peaks in the mass spectra. For the MALDI-MS/MS collision induced dissociation (CID) experiments, the ions of interest were mass-selected by the quadrupole mass filter. The selected ions were then submitted to collision against argon in the T-wave collision cell and the laboratory frame kinetic energy was selected to afford intense enough product ion signals. All the ions exiting the collision cell, either the product ions or the non-dissociated precursor ions, were finally mass measured with the orthogonal-acceleration (oa)-TOF analyzer. Time-of-flight mass analyses were performed in the reflectron mode at a resolution of ~ 10,000. The identification of the saponin ions was performed by comparison between the MS and MSMS data recorded in this work with previous reports (Thanh et al., 2006; Dang et al., 2007; Hua et al., 2009; Van Dyck et al., 2009 & 2010).

Results

As mass spectrometry analyses were realized in the positive ion mode, all the saponin ions correspond to $[M+Na^+]$ ions. Full scan spectra of saponins extracted from fresh body wall are shown in Figure 2 and trepang in Figure 3. All three replicates showed similar mass spectra. Tandem mass spectrometry (not shown) revealed that at least six peaks correspond to saponin ion signals: *m/z* 1125 (holothurinoside C), 1141 (desholothurin A), 1213 (pervicoside C), 1227 (scabraside A), 1243 (scabraside B) and 1449 (holothurinoside G), differing by their aglycone structure, sugar composition and the possible presence of a sulfate group. Their molecular structures are shown in Figure 4. Fresh body walls and trepangs of *H. scabra* therefore seem to present similar saponin contents in terms of number and molecular structure of the congeners detected. However, the relative intensities of the five saponin ions were different suggesting that the proportions of the five congeners differ between the fresh and processed holothuroid tissues (Table 1). In fresh body wall, the major peak was detected at *m/z* 1213 followed by *m/z* 1227, 1243, 1449, 1141 and 1125. In comparison, the saponin ion presenting the highest relative intensity in the mass

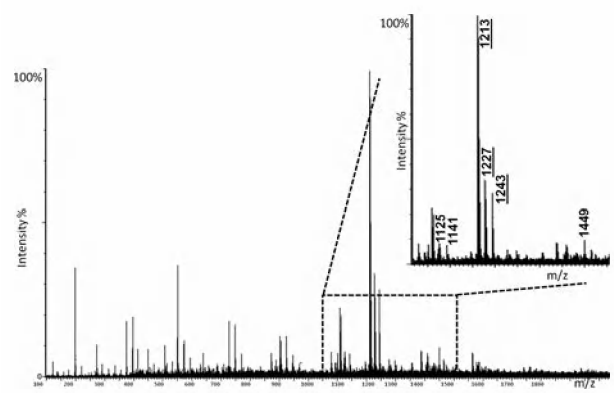


Figure 2: Analysis of saponins extracted from *H.scabra* body-wall by mass spectrometry. Sulfated Saponins are underlined, non-sulfated are not. Most of the other peaks correspond to matrix adducts or plastic contaminants.

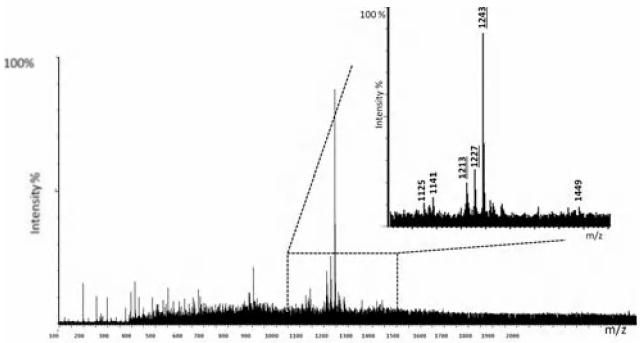
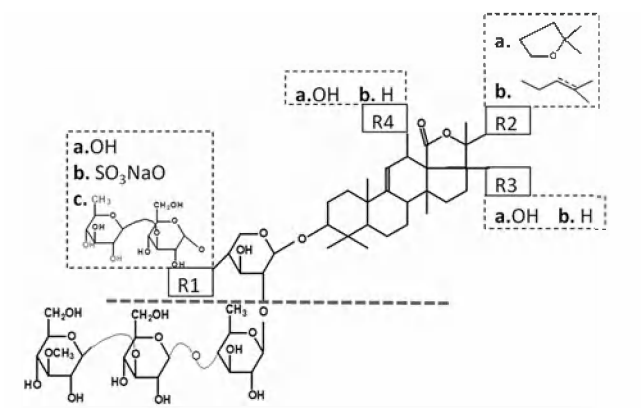


Figure 3: Analysis of saponins extracted from *H. scabra* trepang by mass spectrometry. Sulfated Saponins are underlined, non sulfated are not. Most of the other peaks correspond to matrix adducts or plastic contaminants.

spectrum of the trepang extract was observed at *m/z* 1243, followed by *m/z* 1227, 1213, 1141, 1125 and 1449.

By contrast, no saponin ion was found in the water used to cook the trepangs. Only one major peak was observed at *m/z* 507 in the corresponding MALDI mass spectrum (not shown). Tandem mass spectrometry revealed that this signal corresponds to the trisaccharide methylglucose-glucose-quinovose. This sugar sequence is commonly found in saponins, including in the five congeners detected in *H. scabra* (Fig. 4, in grey line).



Saponins	[M+Na]	R1	R2	R3	R4
Holothurinoside C	1125	a.	a.	b.	b.
Desholothurine A	1141			a.	a.
Pervicoside C	1213	b.	b.	b	a
Scabraside A	1227		a.	b.	b.
Scabraside B	1243	b		a.	a.
Holothurinoside G	1449	c		a.	a.

Figure 4: Hypothetic molecular structure of the six saponins found in the integument of *H.scabra* based on the analysis of their MS/MS spectra. The grey line indicates the place where the hydrolysis occurs in cooked water. Molecular weight [M + Na^+] of the saponins are from 1125 to 1449 and 507 Da for the sugar part after hydrolysis.

Table 1: (+) MALDI-MS full scan analysis: relative intensities (mean ± SD) of saponin signals (M + Na^+) in the body wall and trepang of *Holothuria scabra*. Three individuals were analysed for each type of samples.

Body wall		Trepang	
$[M + Na]^+$	Relative intensity[a]	$[M + Na]^+$	Relative intensity[a]
1125	9.5 ± 1.4	1125	13.4 ± 2.7
1141	8.4 ± 3.4	1141	16.2 ± 8.4
1213	100.0	1213	25.7 ± 7.3
1227	33.1± 4.8	1227	28.6 ± 5.2
1243	29.4± 4.3	1243	100.0
1449	11.6 ± 3.2	1449	9.1± 3.4

[a] % of the most intense peak

Saponin concentrations were calculated as the ratio of the saponin extract dry weight to the body wall wet weight. The mean concentration of saponins was 1.2 ± 0.2 $g.kg^{-1}$ in holothuroid fresh body wall and 0.9 ± 0.3 $g.kg^{-1}$ in the re-hydrated trepang. Statistical test showed no significant differences between those two concentrations (Pvalue > 0.05).

Discussion

The term functional food refers to natural products that offer both nutritional and therapeutic properties to consumers and that are thought to reduce the risk of various chronic diseases (Bordbar et al., 2011). Because they are consumed both for food and for traditional medicine, processed holothuroids, i.e. trepang, are a good example of a marine functional food. Our results showed that trepangs of *H. scabra*, despite the multi-step processing used in their preparation, still contain bioactive saponins, these molecules presumably providing most of the pharmacological properties given to trepangs. Similar saponin ion signals were indeed detected in fresh body wall and in trepang extracts. Moreover, the amount of saponins contained in both tissues is roughly the same, about 1 gram of saponin by kilogram of wet tissue. This concentration corresponds to the saponin concentrations measured by Van Dyck et al. (2010) by semi-quantitative assays in five other Indo-Pacific species from the same family (Holothuriidae). Conversely, no saponin ion was detected in the cooking water but a signal corresponding to the trisaccharide methylglucose-glucose-quinovose was present. Altogether, these results suggest that only a limited amount of saponin is extracted during cooking. However, once dissolved in boiling water, saponins are degraded, presumably by heat. Anyhow, saponins seem to be thermically resistant if they remain in the holothuroid tissues.

In both fresh body wall and in trepang extracts, six major congeners were identified, including three sulfated (scabrasides A and B, pervicoside C) and three non-sulfated saponins (holothurinosides C and G, desholothurin A). The presence of non-sulfated saponins had never been reported in this species. An exhaustive description of saponin content should also be conducted using LC-MS in order to characterize putative isomers (see e.g. Van Dyck et al., 2009). Although the same congeners are present in fresh and processed body walls, the relative intensities of saponin ion signals differ. For instance, the most intense ion in fresh sea cucumbers is pervicoside C (*m/z* 1213) while in trepang it is scabraside B (*m/z* 1243). The hypothesis is that the change in saponin proportions could come from their different localizations in the body wall. Indeed, in the body wall of *Holothuria forskali*, Van Dyck et al. (2011) showed that saponin distribution is almost restricted to the outer and inner epithelia, some congeners being specifically located in the epidermis and others in the mesothelium. This could also be the case in *H. scabra* and, as most of the outer body wall is removed during trepang processing (either by scraping or by enzymatic action), the proportion of saponins located in the peripheral layer of the body wall should be lower in the trepang. Scraping could also favor the dissolution of epidermally-located saponin congeners in cooking water and therefore their hydrolysis. To confirm this hypothesis, saponins could be extracted from non-scraped *H. scabra* trepangs. In future research, attention must be given to analysing saponins from trepangs obtained for other species than *H. scabra*. Also, it would be interesting to dilute pure saponins in different water temperatures to determine the limit temperature that can cause the hydrolysis of these secondary metabolites.

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