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Chemical fingerprinting and phylogenetic mapping of saponin congeners from three tropical holothurian sea cucumbers

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ABSTRACT

Holothurians are sedentary marine organisms known to produce saponins (triterpene glycosides), secondary metabolites exhibiting a wide range of biological activities. In this paper, we investigated the saponin contents of semi-purified and membranolytic HPLC fractionated extracts from the body wall of three species of Holothuriidae as an attempt to examine its chemical diversity in relation to phylogenetic data. MALDI–FTICR MS and nano-HPLC-chip Q-TOF MS were used for mass profiling and isomer separation, respectively giving a unique chemical saponin fingerprint. Moreover, the methods used yield the highest number of congeners. However, saponin concentration, bioactivity and chemical diversity had no apparent relationship. MS fingerprint showed the presence of holothurinosides, which was observed for the first time in other *Holothuria* genera besides the basally positioned *Holothuria forskali*. This congener is proposed to be a primitive character that could be used for taxonomic purposes. The phylogenetic mapping also showed that the glycone part of the compound evolved from non-sulfated hexaosides to sulfated tetraosides, which have higher membranolytic activity and hydrophilicity, the two factors affecting the total ecological activity (i.e. chemical defense) of these compounds. This might be an adaptation to increase the fitness of the organism.

1. Introduction

Sea cucumbers (Class Holothuroidea, Phylum Echinodermata) are slow-moving marine animals preyed upon by fishes, sea stars, gastropods, and crustaceans (Francour, 1997). To counter them, holothurians possess biologically active metabolites called saponins in their body wall, viscera, Cuvierian tubules (Bakus, 1968; Bakus, 1974), and gonads (Matsuno and Ishida, 1969). Saponins possess a triterpene lanosterol aglycone, which has an 18(20)-lactone called holostane and carbohydrate chain containing up to six sugar units of xylose (Xyl), glucose (Glc), quinovose (Qui), and 3-O-methylglucose (MeGlc), and in some cases sulfate groups (Kalinin et al., 1996; Stonik et al., 1999; Kalinin et al., 2005; Kalinin et al., 2008). These compounds have membranolytic action on cellular membranes with (5)-6 unsaturated sterols. They interact to the membrane by forming complexes to modify their structural organization and properties leading to the formation of cellular pores, eventually causing lysis (Kalinin et al., 1996; Popov, 2002, Stonik et al., 1999).

This is the main reason for the wide array of biological activities of saponins including ichthyotoxicity. Sea cucumber extracts can damage the capillaries of fish leading to death (Bakus, 1968; Nigrelli, 1952), thus increasing their overall fitness (Kalinin, 2000). The molecular structure of these compounds was preserved as it also provides internal (breeding regulation) and external (defense against predators, fouling organisms, or space competitors) advantages (Kalinin, 2000). These molecules are widely distributed and are very diverse among holothurians. The diversity is due to the variation on the aglycone and glycone moieties. These include the position of double bonds and the presence of different functional groups (i.e. $-OH$, $-COOH$, $-CH_3$) and lateral groups (i.e. acetoxy, keto groups) in the aglycone and the number of sugar chains and the number and position of sulfate groups in the glycone. Even if the diversity is great, saponins from closely related species still retain the same molecular motif (Kalinin et al., 1996; Stonik et al., 1999). In Holothuriidae alone, 59 types of saponins in 41 species were discovered and chemically described with each species having a specific congener mixture (Caulier et al., 2011). Some congeners are shared within this family (e.g., holothurins A and B). On the one hand, some congeners are very specific to each species, genera or even supergenera which imply that these compounds can be potential chemical taxonomic markers (Moraes et al., 2004; Kalinin et al., 2008; Caulier et al., 2011).

In this study, we determined and compared the mass spectrometric profiles of the body wall of three tropical holothurians species (*Holothuria scabra*, *Holothuria impatiens*, and *Holothuria fuscocinerea*) through matrix-assisted laser desorption/ionization (MALDI)–Fourier

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Table 1
Species from Holothuriidae that were included in the phylogenetic analysis.

Species	GenBank Accession Number	
	16s	cox1
Actinopyga agassizi	JN207496	JN207565
Actinopyga echinites	EU822454	EU848216
Bohadschia argus	AY574870	AY574878
Bohadschia bivittata	AY574873	AY574880
Bohadschia marmorata	AY574877	AY574883
Holothuria arguinensis	GQ214735	GQ214755
Holothuria atra	EU220799	EU220820
Holothuria austrinabassa	EU220797	EU220818
Holothuria cinerascens	JN207554	JN207584
Holothuria dakarensis	EU191979	GQ214752
Holothuria edulis	EU220811	EU220830
Holothuria excellens	EU220796	EU220817
Holothuria floridana	EU220803	EU220822
Holothuria forskali (*1*)	GQ214740	GQ214761
Holothuria forskali (*2*)	EU220798	EU220819
Holothuria fuscocinerea	JN207560	JN207618
Holothuria hilla	JN207515	JN207616
Holothuria impatiens (*1*)	GQ214739	GQ214760
Holothuria impatiens (*2*)	JN207526	JN207632
Holothuria lentiginosa lentigenosa	GQ214733	GQ214753
Holothuria leucospilota	JN207541	JN207617
Holothuria lubrica	JN207497	JN207566
Holothuria mammata	EU191949	GQ214743
Holothuria mexicana	EU220802	EU220821
Holothuria nigralutea	EU220805	EU220824
Holothuria nobilis	EU822441	EU848246
Holothuria polii	EU191981	GQ214759
Holothuria portovallartensis	JN207558	JN207574
Holothuria sanctori	GQ214741	GQ214763
Holothuria scabra	EU822456	FJ971395
Holothuria signata	EU220812	EU220831
Holothuria tubulosa	FJ231192	GQ214748
Holothuria whitmaei	AY509147	EU848245
Pearsonothuria graeffei	EU822440	EU848285
Stichopus chloronotus	EU856692	EU856620
Stichopus herrmanni	EU822451	EU848281
Stichopus horrens	EU822434	EU848282
Stichopus ocellatus	EU220793	EU220814

transform ion cyclotron resonance (FTICR) mass spectrometry (MS) and tandem mass spectrometry (MS/MS) analysis. In addition, nano-high performance liquid chromatography (HPLC)-chip quadropole-time of flight (Q-TOF) MS was used to completely analyze the diversity of isomers. Quantitative assays were also performed to compare the natural volumetric concentrations (NVCs) and its membranolytic activity amongst each other. Insights on the evolution and adaptive role of these compounds in the Holothuriidae were also derived by mapping the saponin types on a constructed phylogenetic tree.

2. Materials and methods

2.1. Sample collection

Three species from Holothuriidae (*H. scabra* Jaeger 1833, *H. fuscocinerea* Jaeger 1833, and *H. impatiens* Forskål 1775) were collected from Bolinao, Pangasinan, Philippines. The organisms were placed together in re-circulating water tanks with sediment. Prior to body wall collection, the sea cucumbers were killed by freezing.

2.2. Extraction and purification of saponins (triterpene glycosides) from the body wall of three holothurians

For each species, five to seven samples were dried using paper towels and these were pooled together to negate any interspecies difference in saponin content. Afterwards, pooled samples for each species were divided into six technical replicates and the volumes of each were determined by water displacement. Considering that marine organisms

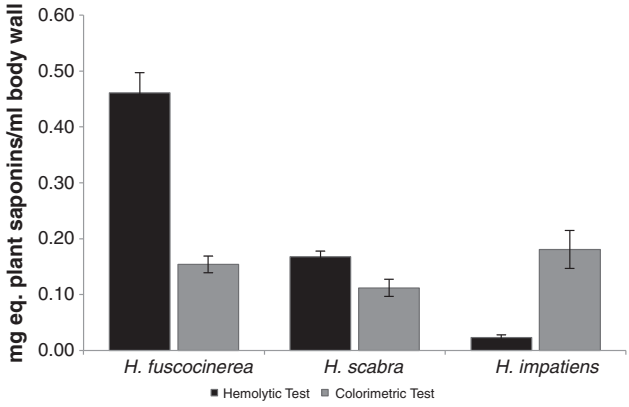

Fig. 1. Quantification of NVC of saponins in the body wall of three holothurian species (n = 6 technical replicates). Values are presented as means ± s.d. and expressed as milligram equivalents of plant saponins per milliliter of body wall.

are highly hydrated, concentrations of chemical compounds produced by marine organisms in natural systems are well reflected by volumetric measurements. Thus, the differences in tissue volume can be controlled (Harvell et al., 1988; O'Neal and Pawlik, 2002).

The extraction methods were modified from those by Van Dyck et al. (2009). The body wall was homogenized and extracted twice with 70% ethanol. The extract was filtered, evaporated under reduced pressure, and lyophilized to obtain a dry extract. The dried extract was re-dissolved in 90% methanol and partitioned with n-hexane (v/v). The water content of the hydromethanolic portion was adjusted to 20% and partitioned against dicholoromethane (v/v). The same phase was adjusted to 40% water content and partitioned against chloroform (v/v). The hydromethanolic phase was then dried and desalted via methanol precipitation. The methanolic portion was evaporated, diluted with water, and partitioned against iso-butanol (v/v). The butanolic portion contained the semi-pure saponins.

The butanolic portions of each species were further purified using reversed-phase HPLC using an HPLC Prominence system equipped with an ultraviolet–visible spectrophotometric detector (Shimadzu, Japan) and a C-18 column (4.6 mm × 150 mm, 5 μm, Waters, Milford, MA, USA). The mobile phase was a nonlinear gradient of 10% methanol (eluent A) and 100% methanol (eluent B). The gradient program was as follows: 0% eluent A at start, 10% to 50% eluent A from 5 min to 20 min, 50% to 85% eluent A from 20 min to 30 min, and back to 10% eluent A from 30 min to 40 min. Fractions were collected every 5 min. The collected fractions were pooled and used for subsequent analysis.

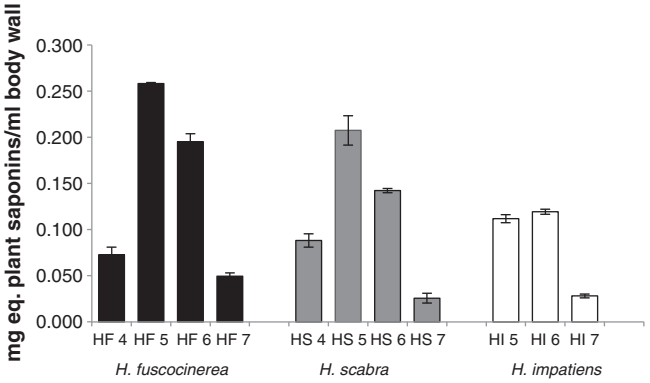

Fig. 2. NVC of HPLC fractions as determined via the hemolytic test (n = 3 technical replicates). Only the active fractions are included in the figure. Values are presented as means ± s.d. and expressed as milligram equivalents of plant saponins per milliliter of body wall.