Process Biochemistry 50 (2015) 867–875

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

In vitro antiviral activities of enzymatic hydrolysates extracted from byproducts of the Atlantic holothurian *Cucumaria frondosa*

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ARTICLE INFO

Article history:
Received 15 December 2014
Received in revised form 10 February 2015
Accepted 23 February 2015
Available online 1 March 2015

Keywords:
Cucumaria frondosa
Enzymatic hydrolysis
Herpes simplex virus type 1
Byproducts

ABSTRACT

Herpes Simplex virus 1 (HSV-1), responsible for the common cold sore, can also lead to serious infections in immunocompromised people. Current antiviral chemotherapies face obstacles including the toxicity of therapeutic molecules, interference with normal cellular metabolism, genetic variability and the incurable nature of latent infections. Therefore, the search for new treatments is a public health issue. Marine invertebrates have held great potential for finding novel antiviral compounds. Little is known, about the antiviral activities of compounds isolated from holothurians. In New Brunswick, holothurian is fished for its edible bodywall and muscle, but its processing generates high amounts of byproducts. *In vitro* evaluation of the anti-HSV-1 activity by cell viability was performed on nine hydrolysates obtained by enzyme-assisted extraction and four solvent extractions from aquapharyngeal bulb and internal organs of *Cucumaria frondosa* at an MOI of 0.001 ID_{50}/cells. After 72 h, four enzymatic hydrolysates from the aquapharyngeal bulb presented effective antiherpetic activities (EC_{50} = 7.2–15.2 μg/mL). After evaluation at a higher MOI (0.01 ID_{50}/cells), the most efficient extract (Papain hydrolysate) was fractionated to identify the active fraction. The fraction superior to 100 kDa showed the highest antiherpetic activity (EC_{50}: 18.2 μg/mL). In conclusion, upgrading byproducts of sea cucumber fisheries offers new sources of bioactive molecules.

1. Introduction

Sea cucumbers are benthic marine invertebrates. Of the 1200 species living worldwide, over 60 are known to be harvested for human consumption [1]. One of the species that is considered new on the market is the orange-footed sea cucumber *Cucumaria frondosa* (Gunnerus, 1767), which is approximately 25–30 cm long and can reach 50 cm when relaxed. The Atlantic sea cucumber *C. frondosa* is distributed abundantly in the north Atlantic from tide pools of the lower intertidal zone and to down to 300 m depth [2].

C. frondosa is harvested in the Fundy Bay, New Brunswick (NB), Canada to be then transformed for the human consumption. The processing plant generates discards representing more than half of the total amount of raw matter (600 t). The waste is mainly used as fish meal or fish oil. Internal muscle bands and the dried body wall are the products from *C. frondosa* sold on Asian food markets. The rest of the sea cucumbers, comprising the aquapharyngeal bulb, gut, gonad, and respiratory tree, considered as byproducts, is rejected or underused. This biomass holds, however, considerable potential to generate new biological compounds and can be turned into a commercially viable business [3,4]. The process of enzymatic hydrolysis has been developed in order to transform marine byproducts into marketable forms. By the use of specific enzymes, this process has the ability to enhance the extraction of specific molecules with new properties (functional, biological, aromatic). Consequently, the action of proteases on marine byproducts rich in proteins may lead to the production of peptides, polypeptides and proteins with various biological activities [3].

Sea cucumbers are a rich source of vitamins, minerals and bioactive components [5]. They are used as healthy foods, traditional medicines and dietary supplements. For example, they have been used for centuries, especially in Asia, as tonic foods and used in folk

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http://dx.doi.org/10.1016/j.procbio.2015.02.012
1359-5113/

medicine to treat various ailments [6]. Dried forms of sea cucumber are used as dietary supplements. More recently, sea cucumbers have received increased attention because of their bioactive compounds such as triterpene glycosides [7–9], chondroitin sulfates [10], sulfated polysaccharides [11], sterols [12], phenolics [13], peptides [14], cerebrosides [15], and lectins [16].

Several studies conducted on holothurians have shown antifungal [17–19] and antibacterial [14,20–22] activities. Little is known about antiviral activities [23–27], especially regarding *Herpes Simplex* Virus 1 (HSV-1) [28–31]. *Herpes simplex* Virus, a DNA enveloped virus, is a common human pathogen with between 60 and up to 95% of certain populations infected with HSV-1, and between 6 and 50% infected with *Herpes Simplex* Virus type 2 (HSV-2). The frequency of HSV-seropositive males was significantly higher in populations infected with Human Immunodeficiency Virus (HIV). As the HIV disease progresses, cutaneous and mucosal complications become more severe and occur in up to 92% of HIV-infected individuals. Medications available for systemic treatment of HSV are acyclovir, famciclovir and valacyclovir. Acyclovir and penciclovir are available for topical use. In clinical practice, treatment of primary HSV infections, while relieving symptoms and reducing the duration of viral shedding, does not prevent recurrences. Moreover, resistance to acyclovir has been reported in immuno-compromised patients. Therefore, there was a need to develop new therapeutic agents for the management of HSV infections. Moreover, the majority of commercially effective molecules are expected to join the public domain in 2015. The search for new treatments is therefore a major public health issue.

The purpose of this study was to evaluate the *in vitro* antiherpetic activities of compounds obtained after enzyme-assisted extraction and solvent extractions of *C. frondosa* (*Dendrochirotida*, *Cucumariidae*) byproducts with a bioguided fractionation.

2. Materials and methods

2.1. Specimen collection

Specimens of *C. frondosa* were collected in Passamaquoddy Bay, Bay of Fundy, New Brunswick, Canada, between January and March 2012. Sea cucumbers (15–30 cm) were placed for acclimation at the Aquaculture Pavilion and Aquaculture Laboratories of the Coastal Zones Research Institute, Shippagan, New Brunswick, in 900 L aerated tanks supplied with filtered seawater (4 °C) pumped from Shippagan's bay, prior to experiments.

2.2. Sample processing

Specimens were sacrificed, then the aquapharyngeal bulb (A.B.) and internal organs (I.O.) were removed from the rest of the sea cucumbers to be separately crushed with a blender (LBC 15, Waring Laboratory Science, USA) and placed at −20 °C prior to further treatments. Some of these samples were freeze-dried and placed in a labeled glass vial at −20 °C prior to the sequential solvent extraction.

2.2.1. Aqueous extraction assisted by enzymatic hydrolysis

For each enzymatic hydrolysis, 1.5 kg of ground biomass was placed in a bioreactor (BioFlo 110, New Brunswick Scientific, USA) with 1.5 L of distilled water. Nine different proteases were separately used: Alcalase (0.1%, *w*/*w*; Alcalase® 2.4 L FG, Novozymes AS, Denmark) with a declared activity of 2.4 AU/g (Anson Unit), Bromelain (0.1%, *w*/*w*; Bio-Cat, Troy, VA, USA) with a declared activity of 2000 GDU/g (Gelatin-Digesting Unit), Flavourzyme (0.1%, *w*/*w*; Flavourzyme® Novozymes AS, Denmark) with a declared activity of 500 LAPU/g (Leucine Amino Peptidase Unit), Fungal Protease (0.1%, *w*/*w*; Bio-Cat, Troy, VA, USA) with a declared activity of 400,000 HU/g (Hemoglobin Unit), Neutral Protease (0.1%, *w*/*w*; Bio-Cat, Troy, VA, USA) with a declared activity of 2,000,000 PC/g (Proteolytic unit), Papain (0.1%, *w*/*w*; Bio-Cat, Troy, VA, USA) with a declared activity of 6500 NFPU/g (Nuclear Floating Power Unit), Peptidase AM (Peptidase *Aspergillus melleus*; 0.1%, *w*/*w*; Bio-Cat, Troy, VA, USA) with a declared activity of 500 LAPU/g, Peptidase AO (Peptidase *Aspergillus oryzae*; 0.1%, *w*/*w*; Bio-Cat, Troy, VA, USA) with a declared activity of 500 LAPU/g and Protamex (0.1%, *w*/*w*; Protamex® Novozymes AS, Denmark) with a declared activity of 1.5 AU/g during 6 h at pH 8.0 and 50 °C. After hydrolysis, enzymes were then inactivated at 90 °C for 15 min and hydrolysates were centrifuged at 10,000 × *g* for 20 min at 4 °C to separate undigested residues and solubilized compounds. The supernatants were sampled, freeze-dried and stored at −20 °C prior to cytotoxicity and antiviral evaluation [14]. The enzymatic hydrolysis process is summarized in Fig. 1.

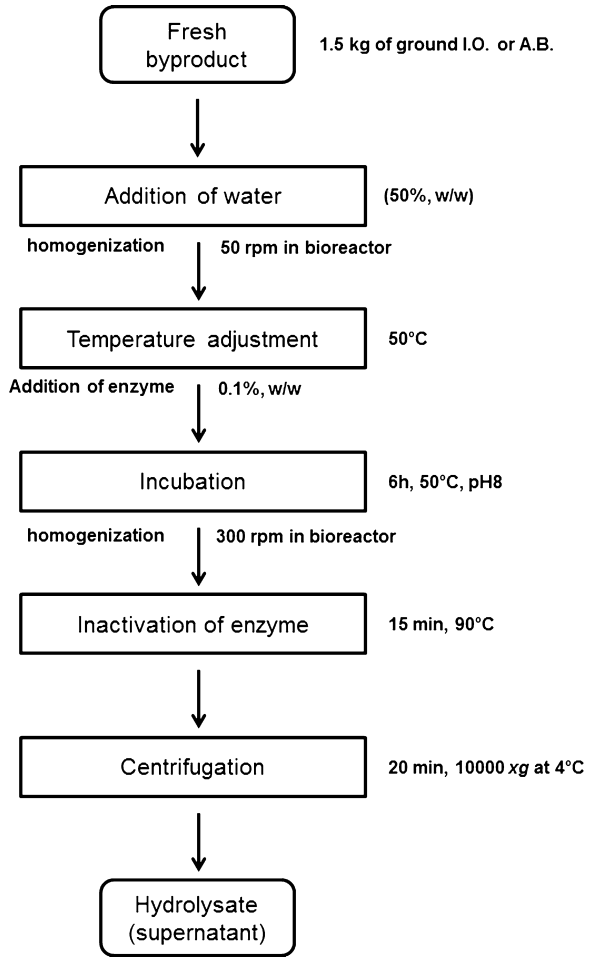

Fig. 1. Summary of the enzymatic hydrolysis process for the preparation of byproducts hydrolysates from *Cucumaria frondosa* (A.B.: aquapharyngeal bulb and I.O.: internal organs); rpm: rotation per minute.

2.2.2. Sequential solvent extraction

Hexane (OmniSolv® Hexanes), acetone (OmniSolv® Acetone) and methanol (OmniSolv® Methanol) are HPLC grade and were provided by Fisher Scientific (Canada). The sequential solvent extraction is summarized in Fig. 2.

Hexane extraction: 20 g of freeze-dried sample of raw matter were first submitted to hexane extraction into a 500 mL Erlenmeyer flask, and 300 mL (15 mL/g) of hexane was added. The sample was placed on a stir-plate, stirred for 30 min at room temperature and