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Inhibitory action of marine algae extracts on the *Trypanosoma cruzi* dihydroorotate dehydrogenase activity and on the protozoan growth in mammalian cells

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Abstract

Trypanosoma cruzi, the causative agent of Chagas' disease, replicates in mammalian cells and relies on the de novo pyrimidine biosynthetic pathway that supplies essential precursors for nucleic acid synthesis. The protozoan dihydroorotate dehydrogenase (DHOD), the fourth enzyme of the pathway catalyzing production of orotate from dihydroorotate, markedly differs from the human enzyme. This study was thus aimed to search for potent inhibitors against *T. cruzi* DHOD activity, and a number of methanol extracts prepared from green, brown, and red algae were assayed. The extracts from two brown algae, *Fucus evanescens* and *Pelvetia babingtonii*, yielded 59 and 58% decrease in the recombinant DHOD activity, respectively, at the concentration of 50 μg/ml. Inhibition by these extracts was noncompetitive with respect to dihydroorotate, with apparent K_i values of 35.3±5.9 and 10.3±4.4 μg/ml, respectively. Further, in an in vitro *T. cruzi*–HeLa cell infection system, ethanol-reconstituted *F. evanescens* and *P. babingtonii* extracts at the concentration of 1 μg/ml, respectively, decreased significantly the infection rate of host cells and the average parasite number per infected cell. These results imply that *F. evanescens* and *P. babingtonii* contain inhibitor(s) against the *T. cruzi* DHOD activity and against the protozoan infection and proliferation in mammalian cells. Identification of inhibitor(s) in these two brown algae and further screening of other marine algae may facilitate the discovery of new, anti-trypanosomal lead compounds.

Keywords: *Trypanosoma cruzi*; De novo pyrimidine biosynthesis; Dihydroorotate dehydrogenase; Marine algae extracts

1. Introduction

Chagas' disease, endemic in Central and South America, is one of the important tropical diseases and is caused by infection of a flagellated protozoan, *Trypanosoma cruzi*, transmitted by sucking bugs. While medication is usually effective when given during the acute phase, once the disease enters into the chronic phase, no medication is proven effective [1]. Therefore, development of chemotherapeutic drugs is urgently needed.

Molecular identification and characterization of enzymes and metabolic pathways that are essential and distinct in *T. cruzi* provide the greater potential of the primary targets for screening a number of bioresources in vitro in the search for a new generation of chemotherapy [2]. Pyrimidine biosynthesis is an essential biological activity for supplying nucleotide precursors to RNA and DNA syntheses in all living organisms and is conducted by the de novo and salvage pathways. *T. cruzi* possesses both the pathways, but the balance between de novo and salvage activities varies in different developmental stages of the parasite; it is likely that amastigotes essentially rely on the de novo pyrimidine biosynthesis in vivo [3]. The de novo pathway comprises six sequential steps of enzymatic

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reactions. Dihydroorotate dehydrogenase (DHOD) is the fourth enzyme that catalyses orotate formation from dihydroorotate and transfers the resulting electrons to appropriate acceptors. Previously, we cloned *T. cruzi DHOD* gene that occurs in the pyrimidine-biosynthetic gene cluster [4]. Phylogenetic analysis of amino acid sequences of various DHODs revealed that *T. cruzi* DHOD differs significantly from human DHOD [5]. The former localizes in the protozoan cytosol and utilizes preferentially fumarate as electron acceptor, while the latter localizes in the inner membrane of mitochondria and uses ubiquinone as electron acceptor [6]. In addition, *T. cruzi* DHOD is responsible for approximately 40% of total cellular fumarate reductase activity. We recently demonstrated that the DHOD gene-knockout *T. cruzi* could not survive even in the presence of pyrimidine precursors, indicating that DHOD is essential in the parasite and plays important roles not only in the de novo pyrimidine biosynthesis but also in the protozoan cellular redox balance [7]. From these views, the parasite DHOD would be a good target for the development of drugs against Chagas' disease.

Recently, marine algae have been highlighted as resources that contain a variety of biologically active compounds, with antibacterial, antitumor, and immunostimulating activities [8–11]. The present study was aimed to screen various marine algae for their inhibitory effects on the recombinant *T. cruzi* DHOD activity. Two methanol extracts from brown algae, *Fucus evanescens*, and *Pelvetia babingtonii*, showed significant decreases in the DHOD activity in noncompetitive manner with respect to the substrate, dihydroorotate. Further, these two extracts yielded significant inhibitions of the *T. cruzi* infection and proliferation in cultured mammalian cells.

2. Materials and methods

2.1. Parasite and host cells

HeLa cells, a human cancer cell line, were infected by the mammalian stages of *T. cruzi* Tulahuen strain [12]. Time courses of *T. cruzi* infection and proliferation in HeLa cells were as described [13]. HeLa cells or the cells infected with *T. cruzi* were inoculated at an initial cell density of $3–5\times10^5$/ml into Eagle's Minimum Essential Medium (MEM, Sigma-Aldrich Japan) supplemented with 10% fetal bovine serum (FBS) in 25-cm^2 culture flasks and subcultured every 3–4 days at 37 °C and at 5% CO_2 in air. Trypomastigotes, the infective form of *T. cruzi*, were collected from the preceding subculture of the infected HeLa cells. The medium of this subculture was recovered into 15-ml polypropylene tubes and centrifuged at $800\times g$ for 5 min at 4 °C to remove the host cells and cell debris. The resulting supernatant was then centrifuged at $1500\times g$ for 10 min at 4 °C and the pellet containing trypomastigotes was washed three times with 10 ml of MEM by repeated suspension and centrifugation. The purified trypomastigotes were counted on an improved Neubauer hemacytometer and verified to contain less than 10% of amastigotes, the replicating form of the parasite. Epimastigotes, the insect stage of *T. cruzi*, were routinely subcultured every 1 week in a serum-free medium (5.0 ml), GIT (Nippon Seiyaku, Tokyo), containing 10 μg/ml of hemin (Sigma-Aldrich Japan) by seeding epimastigotes at an initial density of 5×10^5/ml in tightly capped 25-cm^2 culture flasks at 26 °C.

2.2. Preparation of algae extracts

Seventy-nine different marine algae samples consisting of one seaweed, six green algae, 20 brown algae, and 52 red algae were collected along the coastline of Japan (Table 1). Preparation of algae extracts was essentially as described [14]. Briefly, wet alga sample (5.0 g) was homogenized with four volumes of phosphate-buffered saline (PBS, pH 7.2). After sedimentation by centrifugation, the pellet was suspended in four volumes of methanol by thorough pipetting and filtrated through a disposable filter unit with a pore size of 0.22 μm and stored at −20 °C until use. This preparation is designated as methanol extract. Methanol extracts from two brown algae, *F. evanescens* and *P. babingtonii*, were dried up using an evaporator and the resulting pellets were dissolved in ethanol to give a concentration of 20 mg/ml, followed by storage at −20 °C until use. These are designated as ethanol extracts and used to examine their effects on *T. cruzi* infection and proliferation in cultured mammalian cells.

2.3. Preparation of the recombinant T. cruzi DHOD

Preparation of the recombinant *T. cruzi* DHOD was carried out as described with minor modifications [6]. Briefly, the open reading frame of *T. cruzi DHOD* gene carried by the recombinant phage DNA (clone No. 4, in Ref. [15]) was subcloned into an expression vector, pET28a (Novagen). The strain BL21 (DE3) of *Escherichia coli* (Novagen) was transformed by the resulting plasmid DNA. Induction of expression was carried out by 1 mM isopropyl-β-D-thiogalactopyranoside (Wako Pure Chemical Industries, Tokyo) for 2 h. The recombinant DHOD having His6-tag at its N-terminus was purified from the cytosolic fraction of the bacteria using a column packed with TALON® metal affinity resins (CLONTECH Laboratories). The column was washed with 100 mM NaCl/5 mM imidazole/40 mM Tris, pH 8.0, and the bound proteins were eluted with 100 mM NaCl/100 mM imidazole/40 mM Tris, pH 8.0. This buffer in the protein eluate was exchanged with 50 mM potassium phosphate, pH 8.0, using a PD-10 column (Amersham Bioscience). The purity of this affinity-purified recombinant DHOD was more than 95% when judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The specific activity and kinetic constants of the recombinant DHOD