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Evaluation of μ-Calpain Inhibitory Activity of Korean Indigenous Marine Organism Extracts

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Abstract – Marine organism extracts were prepared from 26 species of Korean indigenous marine organisms, including 25 species belonging in class Anthozoa of phylum Cnidaria and a species belonging to subphylum Urochordata of phylum Chordata, and screened their inhibitory effects against μ-calpain. As a result, the thirteen extracts were found to be active in the criteria of $IC_{50} < 100$ μg/mL. Among them, the MeOH extracts of *Plexauroides praelonga* and *Alveopora japonica* showed remarkable μ-calpain inhibitory activity with IC_{50} values of 4.62 ± 0.22 and 4.82 ± 0.07 μg/mL, respectively. In addition, chemical investigation of *A. japonica* led to the isolation of an active compound, hexadecyl tetradecanoate, as a selective cathepsin B inhibitor ($IC_{50} = 9.05 \pm 2.45$ μM). This compound was isolated as constituent of *A. japonica* for the first time in the present study.
Keywords – Korean indigenous marine organism, coral, *Alveopora japonica*, hexadecyl tetradecanoate, μ-calpain, cathepsin B

Introduction

Calpains are a family of intracellular calcium-dependent cysteine proteases consists of at least 15 isomers, each of which is encoded by an independent gene (Perrin and Huttenlocher, 2002). Some of these are widely expressed with ubiquitous (calpains 1, 2, 4, 5, 7, 10, 12, 14 and 15) and tissue specific (calpains 3, 6, 8, 9, 11 and 13). Among them, the most widely studied isoforms, calpain 1 (μ-calpain) and calpain 2 (m-calapin) are activated by micromolar and millimolar concentrations of calcium ions (Ca^{2+}), respectively (Pietsch *et al.*, 2010). Calpains are implicated in various patho-physiological process (Reverter *et al.*, 2001) and inappropriate regulation of calpain has been associated with several human diseases, such as brain and spinal cord injury, Alzheimer's and Parkinson's diseases, cancer, cataract formation, and so on (Carragher, 2006). Therefore, the involvement of μ-calpain in human patho-physiological disorders has attracted much interest in the identification of calpain inhibitors and their therapeutic potential. Most inhibitors to date are peptide analogues represented by MDL-28170 (Abell *et al*., 2007; Donker *et al*., 2008; Zhang *et al.*, 2009). However, many of the reported calpain inhibitors including MDL-28170 were found to inhibit closely related cysteine proteases, such as cathepsins B and L, because of their poor selectivity. Cathepsins B and L were shown to be active-site related and physiologically relevant to μ-calpain (Cuerrier *et al*., 2007). CA-074 and Z-FF-FMK have been most widely employed as a cathepsin B inhibitor (Mueller-Steiner *et al*., 2006) and cathepsin L inhibitor, respectively (Boland *et al*., 2004).

As part of our ongoing search for the pharmacological agents from Korean indigenous marine organism (Han *et al*., 2005; Youn, *et al*., 2011), the specimens, 25 species belonging in class Anthozoa of phylum Cnidaria and one species belonging to subphylum Urochordata of phylum Chordata, were collected in Jeju island, Korea and extracted by the standard extraction method established for the extract samples for sale at the Korea Coral Resources Bank. In the present study, 26 Korean indigenous marine organism extracts were tested to evaluate their effects on fluorimetric μ-calpain inhibition assay for the first time. Furthermore, the isolation work on *Alveopora japonica* (Poritidae) and the biological evaluation of a lead compound are described herein.

Experimental

Chemical – The solvents and reagents were of the best

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commercial grade available and were used without further purification unless noted. TLC plates were Silica gel 60 F_{254} (Merck, Germany) and silica gel for column chromatography was Silica gel 60 (SiO_2, 230 - 400 mesh, Merck). μ-Calpain (human erythrocyte), cathepsin B and L were purchased from Calbiochem (Darmstadt, Germany). Pep2, a substrate of μ-calpain was synthesized by the Peptron Corp. (Daejeon, Korea). The cathepsin substrates, RR-AMC for cathepsin B and Z-FR-AMC for cathepsin L, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). MDL28170 (known as a μ-calpain inhibitor), CA-074 (known as a cathepsin B inhibitor), and Z-FF-FMK (known as a cathepsin L inhibitor) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animal material – The specimens, 26 species of marine organisms, were collected from Munseom, Beomseom, Supseom of Jeju Island and Geokumdo from November 2008 to July 2010 by SCUBA diving, and identified by one of the authors, Dr. Jun-Im Song (Department of Biological Science, Ewha Womans University). Among them, 25 species belonged in class Anthozoa of phylum Cnidaria and one species, *Herdmania momus* was subphylum Urochordata of phylum Chordata. The voucher specimens with barcode numbers were deposited at the Korea Coral Resources Bank, Ewha Womans University.

Extraction and isolation – Each collected marine organism was washed with cold water quickly to remove a salt contained in its body. After waters off the sample, its whole body was measured its wet weight and grinded. The grinded sample was suspended in MeOH [tree times volume of the grinded sample (v/v)], sonicated for an hour, an hour later, sonicated for an hour again, and then extracted for 20 hour at room temperature, finishing up the third sonication treatment for an hour. After this extraction process repeated tree times for each sample, the extracted sample was filtered and evaporated *in vacuo*, to afford the MeOH-soluble extract. Each sample of extracts was further freeze-dried and weighed 10 mg in the amber wide crimp-top vial with barcode number to produce the standard marine organism extract for parceling it out.

The freeze-dried stony corals, *A. japonica* (1 kg) were grinded and extracted with MeOH three times by sonication method under room temperature. The MeOH solutions were concentrated *in vacuo* to yield a dried MeOH-soluble extract (20 g). This extract was suspended in distilled water and partitioned with hexane, EtOAc, and *n*-BuOH, sequentially. The hexane extract (7 g) was chromatographed over a silica gel (70 g) column, eluting with a gradient solvent system of hexane-EtOAc (100 : 1 to 1 : 1), to afford a compound, hexadecyl tetradecanoate (20.11 mg) and twenty fractions (H1-H20).

Fluorometric μ-calpain inhibition assay – The fluorometric assay was performed in 96-well plates as described previously (Kang *et al.*, 2009). The substrate used was fluorescence-based probe designed to possess the μ-calpain-cleavage site of p35 which was [2-Abz]-Ser-Thr-Phe-Ala-Gln-Pro-[3-nitrotyrosine]-NH_2 named pep2. μ-Calpain specifically cleaves pep2 between phenylalanine and alanine. It consists of a donor fluorescence group and an acceptor moiety that is capable of intramolecular quenching of the donor's fluorescence. The cleavage between phenylalanine and alanine results in the increase in fluorescence intensity and thus indicates μ-calpain activity. The assay was performed in a final volume of 100 μL. Stock solutions of pep2 and inhibitors were prepared in DMSO and stored at −20 ºC. μ-Calpain inhibition was assayed in reaction buffer (50 mM Tris-HCl, 50 mM NaCl. 1 mM EDTA, 1 mM EGTA and 5 mM *β*-mercaptoethanol, pH 7.5) with 100 μM pep2, 2.5 mM $CaCl_2$ and 5.25 units/ml μ-calpain. μ-Calpain inhibition was observed by adding in the order of substrate, μ-calpain, compound, and $CaCl_2$ solution, and finally incubated with shaking at room temperature for 30 min. Fluorescence intensities were measured at 360 nm excitation and 420 nm emission wavelength. The end-point fluorescence intensity in each well was measured using Microplate Fluorescence Reader (FL600, Bio Tek), and the IC_{50} values were obtained using the data graphing software TableCurve 2D (Systat software Inc.). Fluorescence intensity was indicated by Relative fluorescence unit (RFU). RFU was calculated by subtracting the RFU of the control from all other values. To determine percent inhibition, the percent change in RFU between the activity of the enzyme in the presence and absence of the inhibitor was calculated. Resulting RFU in the absence of inhibitor represents 100% enzyme activity.

Results and Discussion

Twenty-six species of marine organisms were extracted with MeOH using by the standard extraction method established for the extract samples for sale at the Korea Coral Resources Bank. In the present study, these samples were tested for their μ-calpain inhibitory activities and MDL28170, known as a μ-calpain inhibitor, were used as positive control in this assay. As judged in the criteria of inhibitory activity with $IC_{50} < 100$ μg/mL, eleven coral extracts were found to be active as shown in Table 1. Two extracts derived from *Plexauroides praelonga* and *Alveopora japonica* displayed potent μ-calpain inhibitory activities

Table 1. Inhibitory activity of marine organism extracts and positive control against μ-calpain

Species name	Family name	IC_{50} (μg/mL)[a]
Dendronephthya castanea	Nephtheidae	63.92 ± 0.11
Dendronephthya gigantean	Nephtheidae	37.90 ± 4.32
Dendronephthya mollis	Nephtheidae	>100
Dendronephthya putteri	Nephtheidae	45.43 ± 2.64
Dendronephthya suensoni	Nephtheidae	88.44 ± 0.63
Dendronephthya spinulosa	Nephtheidae	>100
Scleronephthya gracillimum	Nephtheidae	85.96 ± 1.67
Umbellulifera spiculosa	Nephtheidae	>100
Acalycigorgia grandiflora	Acanthogorgiidae	>100
Anthoplexaura dimorpha	Plexauridae	72.69 ± 4.19
Bebryce thomsoni	Plexauridae	>100
Calicogorgia granulosa	Plexauridae	77.53 ± 3.13
Euplexaura crassa	Plexauridae	>100
Muricella sp.	Plexauridae	>100
Plexauroides praelonga	Plexauridae	4.62 ± 0.22
Villogorgia antillarum	Plexauridae	>100
Entacmaea quadricolor	Actiniidae	88.52 ± 2.04
Nemanthus nitidus	Nemanthidae	>100
Montipora trabeculata	Acroporidae	>100
Psammocora profundacella	Thamnasteriidae	37.33 ± 2.28
Alveopora japonica	Poritidae	4.82 ± 0.07
Myriopathes japonica	Myriopathidae	>100
Myriopathes ulex	Myriopathidae	>100
Myriopathes ulex	Myriopathidae	>100
Zoanthus sp.(type 3)	-	>100
Herdmania momus	Pleurogona	>100
MDL28170 (positive control)		0.273 ± 0.003

[a]Each data point represents mean ± S.D. from three different experiments performed in triplicate.

with IC_{50} values of 4.62 ± 0.22 and 4.82 ± 0.07 μg/mL, respectively, while nine extracts showed from weak to moderate inhibitory activities with IC_{50} values ranging from 37.3 ± 32.28 to 88.52 ± 2.04 μg/mL. The prospective sample, *A. japonica* has been subjected to detailed chemical investigation to isolate its constituents, resulting in the isolation of four known compounds, ergosta-5,24(28)-dien-3β-ol, a mixture of monoacyl glycrols, eicosanoic acid and tetracosanoic acid, thymine, and 2'-deoxythymidine, in our previous study (Youn *et al*., 2011). Furthermore, extensive chemical work on *A. japonica* has been performed and led to the isolation of a known fatty acid, hexadecyl tetradecanoate. The structure of this compound was determined by comparison of its physical and spectroscopic data with those reported previously (Ieda *et al*., 2008). This compound was evaluated for their μ-calpain inhibitory activities over cathepsins B and L, respectively, and was found to inhibit selectively cathepsin B with IC_{50} value of 9.05 ± 2.45 μM (Table 2). Other constituents of *A. japonica* isolated in our previous study were not tested in these assays because of the limited amounts available. On the basis of these results, novel calpain inhibitors might be expected to be isolated from other active marine organism extracts by further study.

The present investigation demonstrated that some corals among Korean indigenous marine organisms collected in Jeju island, Korea, possess μ-calpain inhibitory activity. It is also suggested that hexadecyl tetradecanoate isolated from the active extract of *A. japonica* may considered, at least in part, as a potential lead for the non-peptidic, selective cathepsin B inhibitor. Hexadecyl tetradecanoate has been isolated only from the unidentified marine sponge which was collected from Yesu Dolsan island of Korea (Kim *et al*., 1990), so this is the first report of its isolation from the stony coral, *A. japonica*. Moreover, this is also the first report of the screening of Korean indigenous marine organism extracts for their μ-calpain inhibitory activity and the evaluation of hexadecyl tetradecanoate for its inhibitory activity against μ-calpain and cathepsins B and L. Hexadecyl tetradecanoate has been exhibited its inhibitory activity against cathepsin B selectively. Thus, further studies are required to determine the mechanism(s) responsible for the cathepsin B inhibition by this compound.

Table 2. Inhibitory activity of hexadecyl tetradecanoate and positive controls against μ-calpain and cathepsins B and L

Compounds	Inhibitory activity as IC_{50} (μM)[a]		
	μ-Calpain	Cathepsin B	Cathepsin L
Hexadecyl tetradecanoate	>50	9.05 ± 2.45	>50
MDL28170	0.0835 ± 0.0019	0.0958 ± 0.0045	0.0031 ± 0.0003
CA-074	>50	0.0037 ± 0.0003	>50
Z-FF-FMK	0.3971 ± 0.0003	0.0656 ± 0.0029	0.0837 ± 0.0008

[a]Each data point represents mean ± S.D. from three different experiments performed in triplicate.

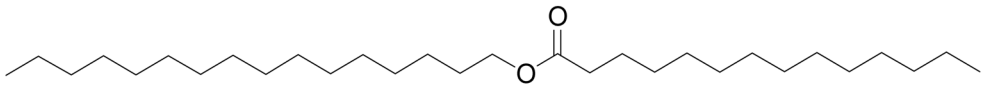

Fig. 1. Chemical structure of hexadecyl tetradecanoate isolated from *A. japonica*.

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