Chapter 21

Purification and Functional Characterization of the Effects on Cell Signaling of Mytilectin: A Novel β-Trefoil Lectin from Marine Mussels

Yuki Fujii, S. M. Abe Kawsar, Imtiaj Hasan, Hideaki Fujita, Marco Gerdol, and Yasuhiro Ozeki

Abstract

In the 2010s, a novel lectin family with β-trefoil folding has been identified in marine mussels from the family Mytilidae (phylum Mollusca). "MytiLec-1," the lectin described in this chapter, was the first member of this family to be isolated and characterized from the Mediterranean mussel *Mytilus galloprovincialis*, a commercially and ecologically important species, spread in marine coastal areas worldwide. MytiLec-1 bound to the sugar moiety of globotriose (Gb3: Galα1-4Galβ1-4Glc), an α-galactoside, leading to apoptosis of Gb3-expressing Burkitt's lymphoma cells. Although the primary structure of MytiLec-1 was quite unusual, its three-dimensional structure was arranged as a β-trefoil fold, which is the typical architecture of "Ricin B chain (or R)-type" lectins, which are found in a broad range of organisms. To date, MytiLec-1-like lectins have been exclusively found in a few species of the mollusk family Mytilidae (*M. galloprovincialis*, *M. trossulus, M. californianus*, and *Crenomytilus grayanus*) and in the phylum Brachiopoda. Transcriptome data revealed the presence of different structural forms of mytilectin in mussels, which included prototype and chimera-type proteins. The primary sequence of these lectins did not match any previously described known protein family, leading to their assignment to the new "mytilectin family." We here report the method of purification of this lectin and describe its use in cell biology.

Key words α-Galactoside, Apoptosis, Caspase, MAPK family, Mytilidae, MytiLec-1, Mytilectin family, Signal transduction

1 Introduction

An α-D-Gal-binding lectin, named "MytiLec" was isolated from the Mediterranean mussel (*Mytilus galloprovincialis*), a highly invasive mytilid species native to the Mediterranean Sea, but now widespread in temperate coastal areas worldwide. MytiLec is a well-investigated protein isolated from this species, which has been characterized on many levels, from the genetic background to 3D structure of the encoded protein. The molecular mass of MytiLec is 17,000 Da per subunit [1]. MytiLec binds strongly to

α-D-Gal, α-D-GalNAc, melibiose, globotriose (Gb3: Galα1-4Galβ1-4Glc) and also weakly binds to *N*-acetyllactosamine (Galβ1-4GlcNAc). Orthologous sequences have been also identified in the congeneric species *Mytilus trossulus* (MTL) [2] and *Mytilus californianus* (MCL) [3], as well as in *Crenomytilus grayanus* (CGL) [4], suggesting that other sequences related with this lectin are likely to be found in other species of the family Mytilidae.

The gene encoding MytiLec consists of two exons, as revealed by alignment between genomic DNA and spliced mRNA sequence (GenBank: FL492661.1 [5]) [6]. Since two other mRNAs encoding proteins similar to MytiLec have been later identified in the mussel transcriptome, we currently often use the term "MytiLec-1" to distinguish this sequence from the others, which have a chimeric structure that includes a C-terminal bacterial toxin-like domain. These lectins did not belong to any previously described protein family and did not show any significant similarity with other sequences deposited in protein databases, and were therefore assigned to a newly named "mytilectin family." The three sequences were further classified as prototype (MytiLec-1) and chimera-type (MytiLec-2/3) lectins, respectively [6]. The MytiLec-1 gene was highly expressed in the mantle and gills of mussels, suggesting that MytiLec-1 plays essential roles in tissues that are in direct contact with the external environment. An acetyl group is added post-translationally at the N-terminal of the translated protein to complete the polypeptide chain.

The primary structure of MytiLec-1 revealed that the polypeptide chain consists of 149 amino acids, with acetyl threonine (Ac-Thr (T)) at the *N*-terminus (B3EWR1 (LEC_MYTGA) [7]) (Fig. 1). BLAST (basic local alignment search tool) searches for homologous proteins revealed >90% homology with orthologous lectins found in other mytilid species and genera. Sequences with ~30% homology were also found in species of the families Pectinidae (Mollusca) and Lingulidae (Brachiopoda) [8]. No MytiLec-like sequences have been found to date in other organisms, including several bivalve species with a fully sequenced genome, such as oysters. Overall, available sequence data indicate that this type of lectin is only present in certain bivalve mollusks and at least in one other phylum (Brachiopoda) within Lophotrochozoa. The primary structure of MytiLec-1 is composed of three repeats of a 50-amino-acid subdomain, which display up to 50% similarity with each other. Each subdomain has acidic amino acids, for example, Asp (D) and Glu (E), on the C-terminal side. A Trp (W) residue is present at the N-terminus of the second subdomain, and the polypeptide contains no Cys (C) residues (Fig. 1).

According to the classification of the protein based on domain identification using the protein family databases Pfam [6], all MytiLec-like sequences in mytilectin family found no significant homology with the canonical signature of the R-type lectin family,

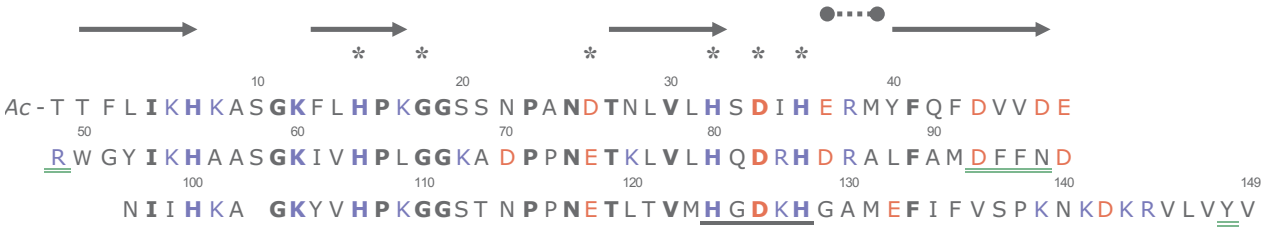

Fig. 1 Primary structure of MytiLec-1. Blue font: basic amino acids. Red font: acidic amino acids. Boldface: amino acids shared by the three subdomains. Underlined (black): HxDxH motifs found in each of the three subdomains, which are essential for binding to C-3, C-4, and C-6 of α-D-Gal/GalNAc. Double underlined (green): amino acids essential for dimerization. Asterisks: amino acids essential for carbohydrate binding. Arrows: β-sheet structure. Dashed line: α-helix structure

nor any QxW motif, which is typically found in all members of the β-trefoil fold superfamily. However, we found the 3D structure of MytiLec-1 involves a β-trefoil fold (3WMV [9]: Research Collaboratory for Structure Bioinformatics Protein Data Bank (RCSB PDB), which is characteristic of ricin B chain (Fig. 2a) [10]. The β-trefoil fold superfamily contains 14 protein families comprising >8000 unique sequences; these include protease inhibitors, fibroblast growth factors, interleukin-1, hemolytic toxins, and numerous R-type lectins [11]. Although MytiLec-1 could not be assigned to any known protein family, it has four conserved β-strands and an α-helix in each subdomain (arrows and dotted line in Fig. 1), similarly to the ricin B chain. Each subdomain of MytiLec-1 contains a functional binding site for glycan, which is unusual (Fig. 2a).

The HxDxH motifs (residues 32–36, 76–80, 134–138) found in each subdomain (black underline in Fig. 1) are essential for binding to C-3, C-4, and C-6 of α-D-Gal/GalNAc (Fig. 2b). The hydroxyl group of the α-anomer of C-1 has a configuration that does not produce steric hindrance. Isothermal titration calorimetry revealed similar dissociation constants (K_d) for Gal (0.2 mM) and GalNAc (0.13 mM) [10]. MytiLec-1 forms a tight noncovalent dimer through a hydrophobic interface comprised mainly of ^{93}Phe and ^{94}Phe (underlined in green in Fig. 1). Overall, MytiLec-1 can be roughly defined as having a "dumbbell" shape, with six carbohydrate-binding sites per molecule (Fig. 2c).

In view of the specific binding of MytiLec-1 to the Gb3 (Galα1-4Galβ1-4Glc1-Cer) oligosaccharide, we investigated the possible effects of MytiLec-1 on growth of human Burkitt's lymphoma cells, which express Gb3 on the cell surface. MytiLec-1 bound to Gb3 oligosaccharide on the cell surface, resulting in turn to the inhibition of cell proliferation. In addition to the cell aggregation, MytiLec-1 appeared to activate MAP kinase and stress-dependent kinases. In response to lectin binding, the expression of kinase-inhibitory protein and tumor necrosis factor was induced, leading to the activation of caspases and induction of apoptosis (programmed cell death) in the target cells [12].

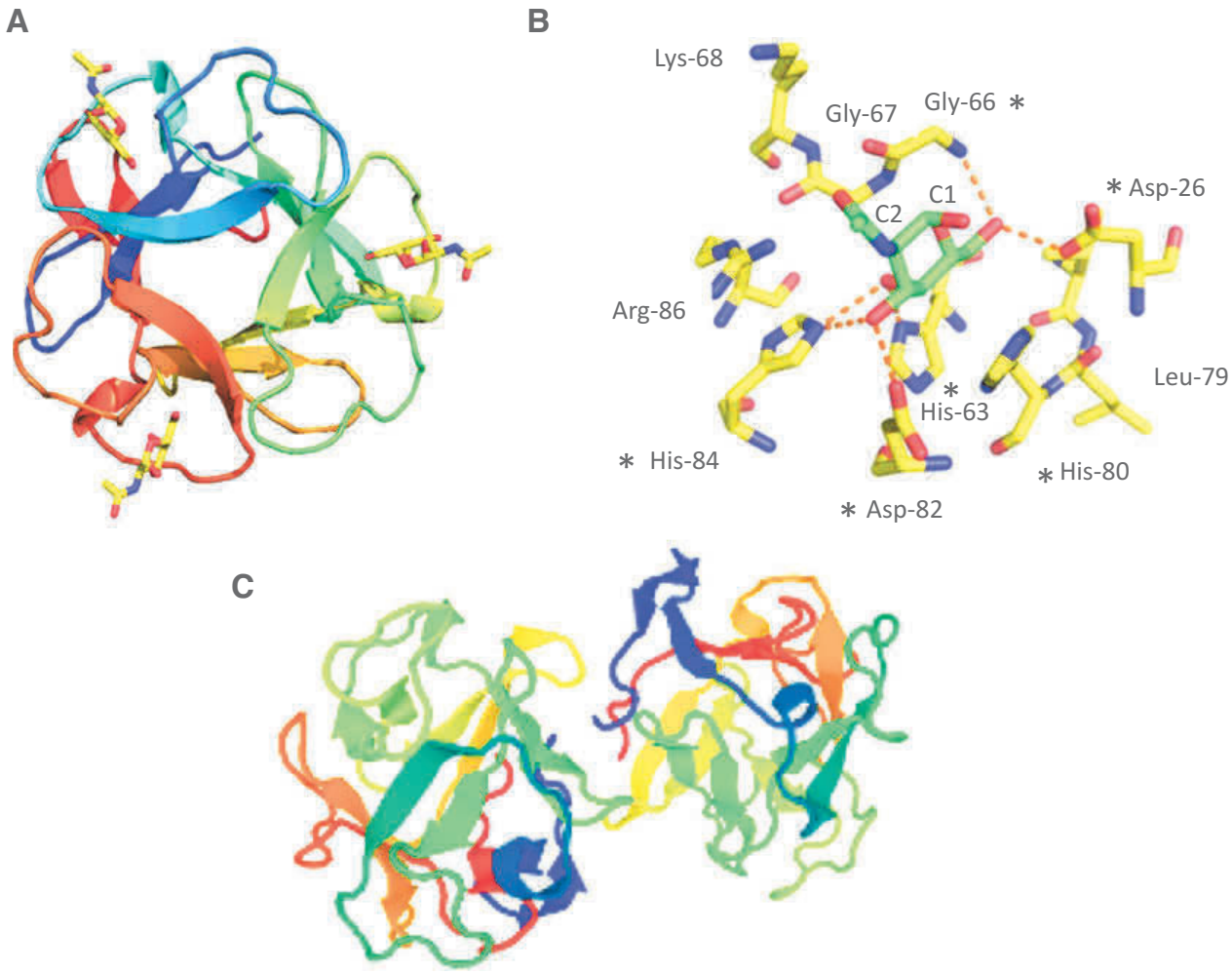

Fig. 2 3D structures of a MytiLec-1 subunit. All subdomains have glycan-binding activity (**a**). Crystallographic analysis indicating the binding of MytiLec-1 to hydroxyl groups at positions C3, C4, and C6 of α-D-GalNAc. The α-anomer direction of the C1 hydroxyl group is compatible with site occupied within the lectin. The lectin did not interact with hydroxyl or N-acetyl group of the C-2 position (**b**). Dumbbell-like dimeric form of MytiLec-1; (**c**)

Many proteins pertaining to the R-type lectin family have accessory functional domains and function as cytotoxins. For example, the antitumor protein pierisin (extracted from larvae of a Japanese butterfly), consisting of a polypeptide chain with three R-type lectin domains and an ADP ribosyltransferase domain, is capable of binding to Gb3 and/or Gb4 (Galβ1-3Galα1-4Galβ1-4Glc) on the surface of HeLa cells, inducing apoptosis through the activity of the enzymatic domain [13]. On the other hand, MytiLec-1, as well as CGL (isolated from *C. grayanus*), lead to the activation of signaling pathways by binding to a Gb3 glycan ligand, directly inducing apoptosis and a modulation of the immune response, respectively [12, 14]. R-type lectins from fungi, consisting only of lectin subunits, can also induce apoptosis [15–17]. In this case, however, the target glycan differs from that of MytiLec-1 or CGL [1, 18]. Indeed, fungal R-type lectins bound preferentially to β-glycoside, whereas the mytilectins bond selectively to α-galactoside.

This chapter provides a detailed description about the purification protocol of MytiLec-1 and further explains how to investigate the effects of this lectin on cell proliferation. Although we describe the growth inhibition of Burkitt's lymphoma cells here, the activity of lectins isolated from invertebrates, plants, and microorganisms on mammalian cells is not only directed toward growth inhibition but has also an effect on proliferation and differentiation [14, 19, 20]. For this reason, the protocol here described will be widely applicable for the study of the functional and pharmacological activities of various lectins.

2 Materials

2.1 Mussels

1. Mediterranean mussels (*Mytilus galloprovincialis*) specimens can be collected from the intertidal zone in rocky seashores (*see* **Note 1**).
2. Keep the mussels in tanks with running seawater or store the sampled organisms at −80 °C until starting the purification protocol (*see* **Note 2**).

2.2 Lectin Purification

1. Melibiosyl-agarose column: 3 mL agarose gel immobilized melibiose (Galα1-6Glc) (EY Laboratories Inc., CA, USA, Catalogue #CG-009-5) in Poly-Prep chromatography column (Bio-Rad Laboratories, CA, USA, Catalogue #731–1550).
2. TBS (Tris buffered saline): 10 mM Tris-HCl and 150 mM NaCl, pH 7.4.
3. D-galactose/TBS: 20 mM D-galactose containing TBS.
4. PBS (phosphate buffered saline): 8 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl, and 137 mM NaCl (pH 7.4).
5. BCA (bicinchoninic acid) Protein Assay Kit (Thermo Fisher Scientific, MA, USA, Catalogue #23225).

2.3 Cell Culture and Cell Growth Assay

1. Raji, Ramos, and K562 cells can be obtained from the American Type Culture Collection (VA, USA) (*see* **Note 3**).
2. Cell culture medium: RPMI 1640 with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.
3. 100-mm culture dishes.
4. 15 mL polypropylene conical tubes.
5. Hemocytometer.
6. 96-wells flat-bottomed plastic plates.

7. Cell Counting Kit-8: WST (water-soluble tetrazolium salt)-8 in 1-metholy-5-methylphenazinium methylsulfate solution (Dojindo Laboratories, Kumamoto, Japan, Catalogue #CK04).
8. Microplate spectrophotometer: Multiskan Microplate Spectrophotometer (Thermo Fisher Scientific, MA, USA).

2.4 Antibodies and Western Blotting

1. Anti-caspase-3 (Cell Signaling Technologies Inc., MA, USA, Catalogue #9662).
2. Anti-caspase-9 (Abcam Cambridge, UK, Catalogue #ab25758).
3. Anti-phospho-$ERK_{1/2}$ antibody (Becton Deckinson & Co, NJ, USA, Catalogue #561991).
4. Anti-$ERK_{1/2}$ antibody (Becton Deckinson & Co, NJ, USA, Catalogue #612358).
5. Anti-phospho-$MEK_{1/2}$ antibody (Cell Signaling Technologies Inc., MA, USA, Catalogue #9121).
6. Anti-$MEK_{1/2}$ antibody (Cell Signaling Technologies Inc., MA, USA, Catalogue CST #9122).
7. RIPA buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, and 1% sodium deoxycholate containing 1× protease inhibitor cocktail (Nacalai Tesque, Inc., Kyoto Japan, Catalogue #25955).
8. PVDF (polyvinylidene difluoride) Membrane P-plus (ATTO Co Ltd. Tokyo, Japan, Catalogue #WSE-4053).
9. Semidry blotting system (ATTO Co Ltd. Tokyo, Japan, Catalogue #WSE-4115).
10. EzFastBlot: Transfer reagent for PVDF membrane (ATTO Co Ltd. Tokyo, Japan, Catalogue #AE-1465).
11. EzBlock Chemi: Blocking reagent for PVDF membrane (ATTO Co Ltd. Tokyo, Japan, Catalogue #AE-1475) (*see* **Note 4**).
12. PBS-T: PBS containing 0.1% (w/v) Triton X-100.
13. 1% (w/v) BSA (bovine serum albumin) in PBS (Thermo Fisher Scientific, MA, USA, catalogue #37525).
14. ECL (enhanced chemical-luminescence) Western Blotting Substrate (Thermo Fisher Scientific, MA, USA, Catalogue #32106).
15. Amersham Imager 680: luminescence image analyzer (GE Healthcare, IL, USA).
16. EzWest Blue (ATTO Co Ltd. Tokyo, Japan, Catalogue #AE-1490).

3 Methods

3.1 Purification of MytiLec-1

1. Open the two valves of the mussel shells by using a single-edged razor to dissect organs with scissors (*see* **Note 5**).
2. Excise mantles and gills, then store them in a petri dish to be frozen at −80 °C (*see* **Note 6**).
3. Slice the tissues into smaller pieces by using single-edged razor and, upon thawing, freeze again the material as explained above. Repeat the cutting procedure until the processed tissue assumes a putty, clay-like consistency (*see* **Note 7**).
4. Homogenize with 10 volumes (w/v) TBS (*see* **Note 8**).
5. Collect supernatant (Sup 1) by centrifugation at 27,500 × *g* for 1 h at 4 °C.
6. Homogenize the precipitate again with 10 volumes (w/v) D-galactose/TBS for 1 h at 4 °C.
7. Centrifuge as in **step 5** to collect supernatant (Sup 2).
8. Dialyze sup 2 extensively against TBS (*see* **Note 9**).
9. Collect both Sup 1 and Sup 2 together and centrifuge again as in **step 5** to remove debris (*see* **Note 10**).
10. Apply the clear supernatant of the crude extract onto a melibiosyl-agarose column and discard the part of the crude extract that washes through the column.
11. Wash the affinity column with TBS until the absorbance of the eluate at 280 nm (A_{280}) reaches baseline level (*see* **Note 11**).
12. Elute MytiLec-1 from the column with D-galactose/TBS (*see* **Note 12**) (Fig. 3).
13. Collect the fractions containing MytiLec-1, detected by SDS-PAGE, and dialyze them with a buffer, such as TBS or PBS.
14. Measure the concentration of the purified lectin with a BCA Protein Assay Kit [21, 22].

3.2 Cell Growth Quantification for MytiLec-1 Treated Cells

1. Grow the culture cells (Raji and K562) to more than 1×10^6 in 100 mm plastic plate.
2. Place the cell suspension in a 15 mL polypropylene conical tube and centrifuge at 200 × *g* at room temperature.
3. Discard the supernatant and resuspend the cells with 5 mL of medium.
4. Count the cell number with a hemocytometer to adjust their density to 1×10^4 in 90 μL/well.

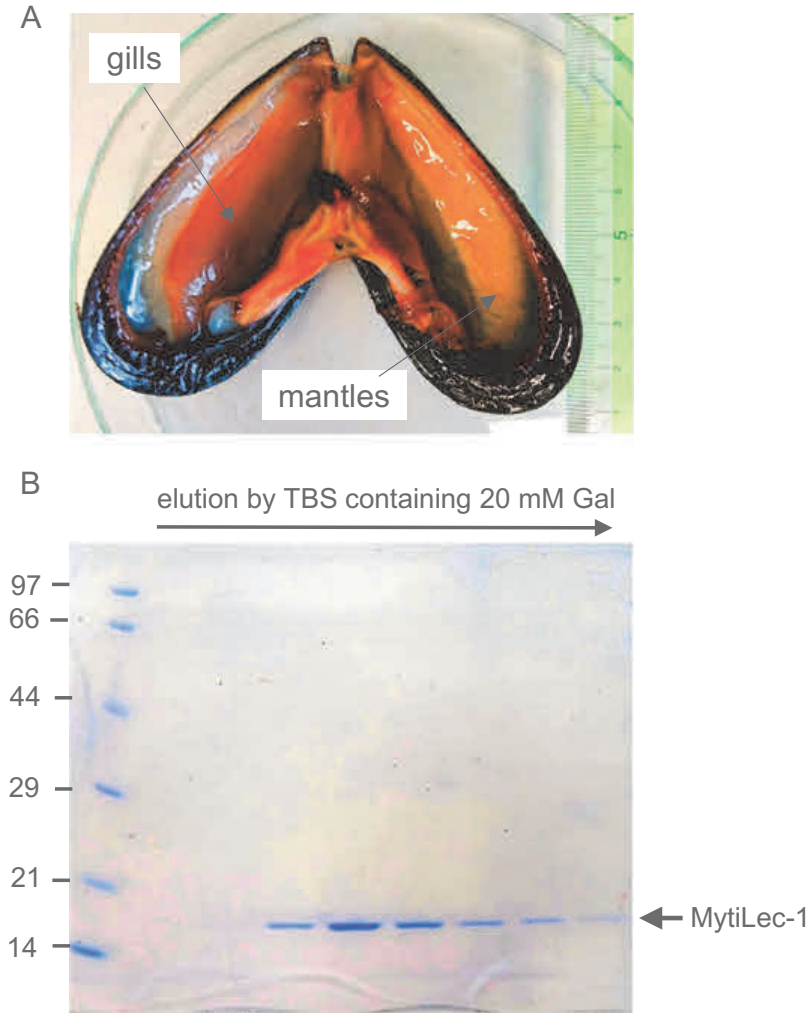

Fig. 3 Purification of MytiLec-1. Localization of mantles and gills of the Mediterranean mussel (*M. galloprovincialis*) (**a**). Elution pattern of MytiLec-1 from melibiosyl-agarose column (5 mL) with TBS containing 20 mM D-Gal. Fractions of 1 mL volume each were collected and separated by SDS-PAGE. Markers: 97 kDa phosphorylase b(rabbit), 66 kDa serum albumin (bovine), 44 kDa ovalbumin (avian), 29 kDa carbonic anhydrase (human), 20 kDa trypsin inhibitor (soybean), and 14 kDa lysozyme (avian egg) (**b**)

5. Seed 1×10^4 cells (90 μL) with several concentrations (0–100 μg/mL) of MytiLec-1 (10 μL) on each well in the 96-wells flat-bottomed plastic plate (*see* **Note 13**).
6. Incubate the plate at 37 °C in the incubator with 5% CO_2 for 24 h.
7. Add WST-8 in 1-metholy-5-methylphenazinium methylsulfate solution (10 μL) of Cell Counting Kit-8 in each well to quantify the ratio of live cells affected by MytiLec-1 and incubate with 5% CO_2 for 4 h at 37 °C (*see* **Note 14**).
8. Measure the absorbance at 450 nm in each well with a microplate spectrophotometer (Fig. 4).

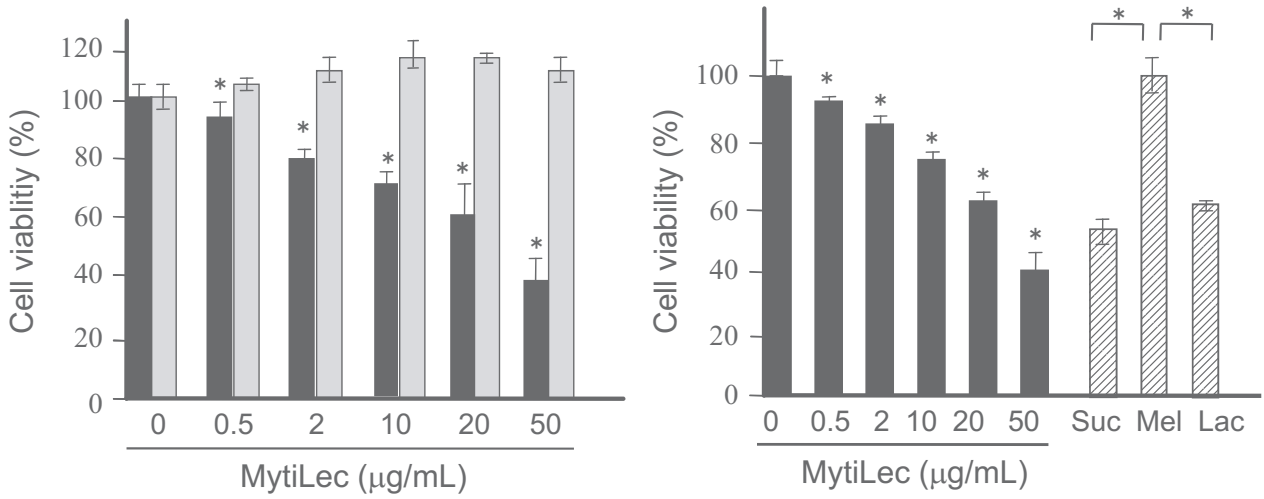

Fig. 4 Glycan-mediated cytotoxic effect of MytiLec-1 on Burkitt's lymphoma Raji cells. Cell viability was determined using a WST-8 assay kit. Raji cells were treated with various concentrations (0–50 μg/mL) of MytiLec-1. Left: viability (absorbance) of Raji cells (black bars) and erythroleukemia K562 cells (gray bars). Right: inhibitory effects of saccharides (Suc: sucrose; Mel: melibiose; Lac: lactose; each 100 mM; gray bars) on reduction of cell viability caused by various concentrations of MytiLec-1 (black bars). Error bars: S.E. ($n = 3$)

3.3 Lectin-Dependent Signal Transduction

1. Grow Burkitt's lymphoma cells (Ramos) to more than 1×10^6 in 100 mm plastic plates.
2. Wash cells as in **steps 2** and **3**, Subheading 3.2.
3. Count the cell number with a hemocytometer to adjust their density to 5×10^5 in 2.7 mL/well.
4. Seed 5×10^5 (2.7 mL) with several concentration (0–100 μg/mL) of MytiLec-1 (300 μL) on each well in the 6-wells culture plate (*see* **Note 13**).
5. Incubate the plate at 37 °C in the 5% CO_2 incubator for 24 h.
6. Collect the cell suspension in each well to 15 mL polypropylene conical tube and centrifuge at 200 × *g* at room temperature, respectively (*see* **Note 15**).
7. Remove the medium and add the PBS (1 mL) to wash the cells.
8. Centrifuge at 200 × *g* at room temperature and repeat the washing procedure twice.
9. After the removal of PBS, add the cell lysis (RIPA) buffer (100 μL) and incubate for 15 min on ice.
10. Extract the cell component by pipetting several times.
11. Apply the cell extract (20 μg) on each well of SDS-PAGE after measurement of the protein concentration with a BCA Protein Assay Kit [21, 22].
12. Separate proteins of the crude cell extraction by electrophoresis, and transfer them onto a PVDF membrane.

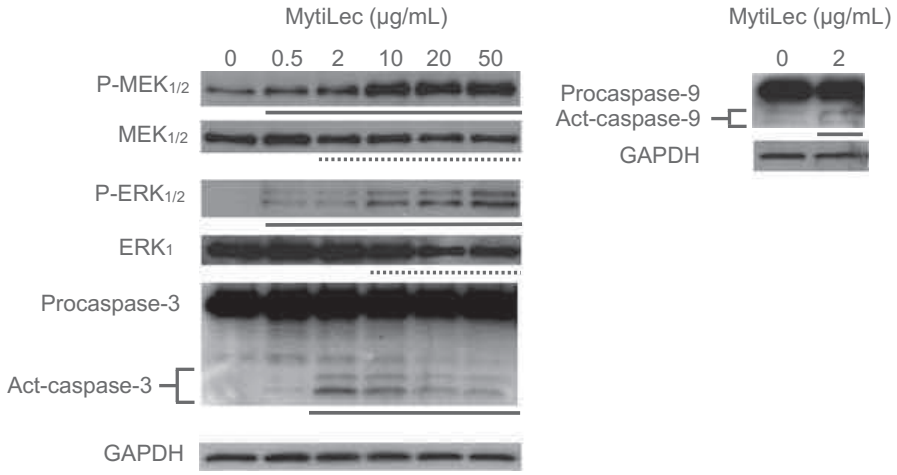

Fig. 5 Effects of MytiLec-1 treatment on MEK and ERK in Burkitt's lymphoma Ramos cells. Phosphorylation levels of MEK1/2 and ERK1/2. P-MEK and P-ERK are activated forms of MEK and ERK, respectively. Act-caspase-3 and act-caspase-9 are activated form of caspase-3 and 9, respectively. GAPDH: glyceraldehyde-3-phosphate dehydrogenase as internal control. The solid line and the dashed line represent increase and decrease of signals, respectively

13. After masking the membrane with blocking reagent EzBlock Chemi for 1 h, incubate it overnight with primary antibodies (1/1000 dilution with 1% BSA in PBS) to detect the activated kinase or caspase, respectively.
14. Wash the PVDF membrane with PBS-T for 5 min and repeat this procedure for three times.
15. Incubate the secondary antibody (1/1000 dilution with 1% BSA in PBS) of each which conjugated with horseradish peroxidase for 2 h at room temperature.
16. Enhance the signal of peroxidase by using ELC Western Blotting Substrate and detect the signal with a luminescence image analyzer, such as Amersham Imager 680 (*see* **Note 16**) (Fig. 5).

4 Notes

1. In addition to *Mytilus galloprovincialis*, which produces MytiLec-1, and other congeneric species where orthologous sequences have been previously described, several morphologically similar mussel species, pertaining to different taxa (e.g., *M. edulis*, *M. coruscus*, *Perna viridis*, and *Mytilisepta virgata*) can be found in the same coastal regions, depending on the geographical location of sampling. The presence of lectins equivalent to MytiLec-1 has not been confirmed yet in these species at the protein level [23].

2. Mussels can be farmed under controlled laboratory conditions for about 3 months up to half a year, by placing them in a low-temperature room (about 15 to 20 °C), in aerated tanks containing artificial seawater, which should be periodically replaced. Liquid food for invertebrates and coral, available for purchase at pet shops, can be used as a source of nutrients for mussels.
3. In addition to Burkitt lymphoma cell line Raji and Ramos cells [24], HeLa (cervical cancer) [13] and Caco-2 (colorectal cancer) cells [25] are also known to express the Gb3 glycan on their cell surface. K562 can be used as negative control, as this cell line does not express this glycan [26].
4. If this reagent is not available, 1% BSA containing PBS (or TBS) can be used as a replacement.
5. Insert the razor near the space where the hinge of the shell is located and cut the adductor muscles to open the shells.
6. This process is effective to grind the tissue to be minced.
7. Grind the tissue when it is in a frozen state with a razor, without adding the buffer.
8. It is not necessary to add divalent cations for the purification of lectins pertaining to this family.
9. The majority of MytiLec-1 is expected to be contained in Sup 2.
10. The crude supernatant should be centrifuged at least three times to completely remove debris and avoid blocking the flow through the column.
11. If a UV photometer is not used for the detection, the column should be washed with a sufficiently high amount of washing buffer, that is, more than 20 times the volume of the column.
12. Once the washing buffer (TBS) inside the column has passed through the column, prepare about ten test tubes. For each test tube, add 1 mL elution buffer (D-galactose/TBS) to the column with a micropipette, and recover the liquid dropped from the outlet of the column.
13. The purified lectin, solved in PBS, should be centrifuged and filtrated with a sterile disposable 0.22 μm pore filter membrane (nonabsorption type).
14. The details of the WST-8 assay are mentioned in the instruction manual provided by the manufacturer.
15. In the case of adhesive cells, seed cells at 50% confluence in a six-well culture plate, in order to achieve almost complete confluence on the day of treatment.

16. The immunocomplex can be also used to perform a qualitative detection by staining the membrane with the substrate of peroxidase, such as EzWest Blue.

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