Characterization of a novel proteinous toxin from sea anemone *Actineria villosa*

Gen-ichiro Uechi[1*], Hiromu Toma[2], Takeshi Arakawa[3], and Yoshiya Sato[2]

[1]Department of Virology, Institute of Tropical Medicine, Nagasaki University, Japan

[2]Division of Tropical Parasitology, Unit of Social and Environmental Medicine, School of Medicine, University of the Ryukyus, Japan

[3]Division of Molecular Microbiology, Center of Molecular Biosciences, University of the Ryukyus, Japan

[*]Corresponding author: Gen-ichiro Uechi, Department of Virology, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki City, Nagasaki 852-8523, Japan

Tel: +81-95-819-7876

Fax: +81-95-819-7830

E-mail: uechi@nagasaki-u.ac.jp

Abstract

The sea anemone *Actineria villosa* expresses a lethal protein toxin. We isolated a novel 120-kDa protein, Avt120, from partially purified toxin and found it to possess extremely strong lethal activity. The 3453-bp Avt120 gene translates to a 995-amino acid protein. The 50% lethal dose (LD_{50}) of purified Avt120 in mice was 85.17 ng. Among several tested cell lines, Colo205 cells were most sensitive to Avt120: 50% of them were damaged by 38.4 ng/ml Avt120. Avt120 exerted ATP degradation activity (10 μmol ATP·h^{-1}·mg^{-1}), which was strongly inhibited by ganglioside GM1 to decrease the cytotoxicity of Avt120.

Keywords: *Actineria villosa,* Avt120, ATP degradation activity, ganglioside, cytotoxic

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
DTT	Dithiothreitol
FCS	Fetal calf serum
HPLC	High performance liquid chromatography
LD	Lethal dose
MEM	Minimal essential medium
MTT assay	Methylthiazole tetrazolium assay
PBS	Phosphate buffered saline
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

1. Introduction

The sea anemone *Actineria villosa* belongs to the family Aliciidae and is closely related to *Phyllodiscus semoni*, which inhabits the sea around Okinawa, Japan. *A. villosa* and *P. semoni* show morphological differences, but both possess globular vesicles on their surface for protection from predators. The composition of toxins found in their nematocysts and their genetic sequences show strong homology. The genetic sequences of the hemolytic toxins Avt-I and Avt-II and Pst-I and Pstx20 purified from *A. villosa* and *P. semoni*, respectively, were 99% homology [13,20,19].

These hemolytic toxins are actinoporins and homologous to other sea anemone hemolytic toxins. The gene encoding Avt-I and Avt-II contains an intron, indicating the unique developmental process of *A. villosa* [18]. PsTX-T purified from *P. semoni* induces renal disorders by binding to glomeruli [12]. Cholesterol inhibits the activity of metridiolysin, a hemolytic toxin from *Metridium senile* [1]. Two cytotoxins (95 and 31 kDa) isolated using a ConA-Sepharose column have been purified from *Aiptasia mutabilis* [11]. However, their mechanisms of action remain unclear.

Actineria villosa crude toxin has a 50% lethal dose (LD_{50}) of 0.01 mg/kg for mice [14]. Crude *A. villosa* toxin appeared to induce lethality by paralyzing animals' muscles (Uechi, unpublished data). Here, we purified the protein toxin with the highest lethal activity from this partially purified fraction.

2. Materials and Methods

2.1 Animals and sample collection

A. villosa was collected along the Odo coast in Itoman-city, Okinawa, Japan. Globular vesicles and surrounding nematocysts were removed from the body surface by

using forceps, and immediately submerged in 10 mM sodium phosphate buffer (pH 6.0) in centrifuge tubes.

2.2 N-terminal amino acid sequence determination

The purified toxin was separated by SDS-PAGE [10]. Purified toxin solution (20 μL, 1 μg/μl) was applied to well and overlaid with 3 U *Staphylococcus aureus* V8 protease (Sigma). The purified toxin was digested in the gel at room temperature for 1 h and then electrophoretically blotted onto a polyvinylidene difluoride membrane (Bio-Rad). The toxin was stained with Coomassie Brilliant Blue, and the band at the 23-kDa position was excised to extract the protein. Auto-proteolytic sample of V8 protease was separated in same gel as negative control. The N-terminal sequence of the digested protein was determined with a PPSQ-21 protein sequencer (Shimadzu). The sequence was checked and confirmed with V8 protease sequence to remove contamination.

2.3 Mice 50% lethal dose analysis

ICR mice were purchased from an Okinawa distributor. The LD_{50} of the toxin was determined in 16 ICR mice (20–25 g), which were intravenously injected with the toxin (200, 100, 50, or 0 ng, 4 mice/group) diluted in 100 μL phosphate buffered saline (PBS, 3.2mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.4) and observed after 24 h to 7 days after injection. LD_{50} was determined by the probit method [5]. All animal experiments were approved by the Institutional Animal Care and Use Committee of Ryukyu University.

2.4 Cytotoxicity assay

Cell strains (HL60, NIH/3T3, Vero, HEK293T, and Colo205) were obtained from the Cell Resource Center for Biomedical Research, Tohoku University. MTT assays of control and toxin-treated cells cultured in 12-well plates were performed using the manufacturer's protocol (Dojindo) to confirm cytotoxic effect. Cells (1×10^5) cultured in MEM medium containing 5% FCS were washed twice in PBS and then incubated at 37°C for 1 h in the presence of 5 μg Avt120 diluted in 100 μl PBS containing 1% bovine serum albumin. Lytic samples used as positive controls were obtained by 0.1% Tween20 treatment. The extent of cytotoxicity was expressed as percent change in MTT assay O.D. values. The cytotoxicity increasing or decreasing of Avt120 against Colo205 cell was measured by addition of 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 3 mM EDTA (pH 8.0).

2.5 ATP degradation assay

ADP and ATP were purchased from Wako (Osaka, Japan). All other chemicals were analytical or HPLC grade. For HPLC, reversed-phase columns (TSK-Gel ODS-100V, 4.6 mm × 25 cm) were obtained from Tosoh (Tokyo, Japan). ATP degradation activity was measured at 25°C in 100 μl solution containing 0.05 M KCl, 20 mM Tris-maleate (pH 7.0), 1 mM ATP, and 5 μg purified Avt120. Reaction was stopped by adding 0.1 ml 10% trichloroacetic acid and centrifugation at 13,000 rpm. ATP degradation was analyzed by reversed-phase HPLC. HPLC conditions were as follows: mobile phase, 100 mM potassium dihydrogen phosphate (pH 4.5); flow rate, 1.0 ml/min; UV detection wavelength, 260 nm; and injection volume, 10 μl.

2.6 ATP degradation inhibition by gangliosides

ATP degradation and cytotoxic inhibition was measured by 1 h pre-incubation of 1 μM GM1, GM2, GM3, GD1a, GD1b, GD2, GT1b, LysoGM1, Asialo GM1, Asialo GM2 ganglioside, ganglioside mixture, sucrose, glucose, fructose, phosphatidylcholine, phosphatidylserine, sphingomyelin, cholesterol, linoleic acid, arachidonic acid and 0.5% TritonX-100 with 5 μg Avt120 at 37°C. Inhibition test was analyzed twice and ATP degradation activity results were averaged. Relative inhibition activity was calculated as compared with an inhibitor-untreated control.

3. Results

3.1 Avt120 purification

A purified fraction (1.2 mg) was obtained from the *A. villosa* crude toxin (20 mg) by HPLC and named Avt120 (Table 1). Avt120 has the highest molecular weight (120 kDa) among the major proteins found in the crude toxin fraction (Fig. 1). Isoelectric focusing analysis showed that the protein had an isoelectric point of 6.5.

3.2 Avt120 gene cloning

The Avt120 gene was cloned by PCR using a degenerate primer constructed according to the internal protein sequence. The unknown 5′-end sequence was cloned using rapid amplification of cDNA ends (RACE) to finally obtain the full-length gene, whose sequence was determined. The 5′- and 3′-end untranslated regions were 112 and 353 bp long, respectively, and the translated region was 2988 bp long (Fig. 2). The GenBank accession number is AB576860. The sequence was used to estimate amino acid and preprosequence lengths or 995 and 20 amino acids, respectively. The molecular weight (108,856.93 Da) and isoelectric point (6.37) of mature protein were calculated by ExPASy Proteomics Server (http://www.expasy.org/tools/pi_tool.html).

Eight cysteine residues were found in the mature protein sequence and cysteine residues may form internal S-S bond because native and DTT-treated Avt120 was observed as monomer in gel filtration column analysis. At only 9 tryptophan residues among 995 amino acids, the tryptophan content was relatively lower than that of other amino acids. A FASTA search (http://fasta.genome.ad.jp/) revealed 26.8% homology with a *Nematostella vectensis* protein (Starlet sea anemone, RefSeq accession number: XP_001636750, locus tag: NEMVE_v1g240898), which consists of 794 amino acids (Fig. 3). Analysis using the PFAM database (http://pfam.sanger.ac.uk/) revealed an ATP-binding motif in the *N. vectensis* protein. Scan analysis for the prosite motif revealed homology with Avt120 and the presence of Na^+/K^+-ATPase beta chain motif in PFAM database.

3.3 Lethal activity against mice

All ICR mice injected intravenously with 200 ng Avt120 died within 24 h. Injection with 100 ng and 50 ng Avt120 resulted in 50% (2/4) and 25% (1/4) lethality, respectively (Table 2). The LD_{50} was 85.17 ng. Trembling was a characteristic systemic sign in toxin-injected mice. Intramuscular toxin injection induced muscular paralysis, twitching, and difficulty in ambulation. Mice died within 1 min when 10 ng Avt120 was injected intracerebrally (data not shown).

3.4 Cellular toxicity

The lethal concentration (LC_{50}) of Avt120 against HL60, NIH/3T3, Vero, HEK293T, and Colo205 cells was 155.6, 211.5, 160.7, 102.2, and 38.4 ng/ml, respectively (Data not shown). Human colon cancer-derived cells, Colo205, enlarged

(up to about 10-fold) after toxin treatment, and the toxic effect could be easily determined. Addition of $MgCl_2$ (1 mM) and $CaCl_2$ (0.2 mM) increased the cytotoxic effect of Avt120 by 9.5% and 25.5%, respectively. Addition of 3 mM EDTA resulted in 95.7% inhibition of Avt120 cytotoxicity (Fig. 4). These results indicate that metal ion including magnesium and calcium were necessary for Avt120 cytotoxicity.

3.5 ATP degradation activity

Avt120 failed to exert phospholipase activity, lipoxygenase activity, proteinase inhibitory activity, hemolytic activity, or inhibitory activity against protein synthesis. We found that 5 μg Avt120 converted 1 mM ATP into ADP in 2 h at 37°C. The converted ADP was detected by reverse phase HPLC system. The ATP degradation activity of Avt120 was 10 μmol $ATP \cdot h^{-1} \cdot mg^{-1}$ (Data not shown).

3.6 Inhibition of ATP degradation activity

Pre-incubation of 1 μM GM1 and lysoGM1 resulted in 84.0% and 87.4% inhibition, respectively, of Avt120 ATP degradation activity (Fig. 5). Another gangliosides, sugars, phospholipids, cholesterol and 0.5% TritonX-100 showed relatively weak inhibition activity. In Colo205 cells, pre-incubation of 1 μM GM1 or lysoGM1 resulted in 77.0% and 80.9% inhibition, respectively, of Avt120 cytotoxicity (Fig. 6).

4. Discussion

The sea anemone *Actineria villosa* induces necrosis in human skin and even renal failure in severe cases. Mizuno *et al*. successfully purified the PsTX-115 toxin

from the crude PsTX-T toxin of *P. semoni* and found that it induced nephropathy via complement activation [12]. The PsTX-115 amino acid sequence is partly identical to that of the Avt120 molecule cloned by us, suggesting that PsTX-115 and Avt120 are homologs with similar activity.

Mice died when intracerebrally injected with a low concentration of Avt120, and mouse brain homogenate showed strong inhibition of lethality when pre-incubated with Avt120 (data not shown). We believe that some components of nerve cells may interact with Avt120. Gangliosides are glycosphingolipids involved in nerve cell signal transduction. The mixture of purified GM1 and lysoGM1 inhibited lethality and ATP degradation activity of Avt120. Avt120 may inhibit or perturb nerve cells, thereby eliciting paralysis.

Many gangliosides inhibit the NADase activity of CD38, the toxic effect of pertussis toxin, and the ADP-ribosyltransferase activity of the exoenzyme C3 [6,7]. Chethankumar *et al*. found that ganglioside GM1 inhibited the phospholipase activity of *Naja naja* venom by 81% and decreased its cytotoxicity against erythrocytes [4]. Moreover, GM1 inhibits arachidonic acid release by mellitin or maitotoxin [2]. Ganglioside Gt1b inhibited the hemolytic activity and lethal effect of echotoxin, from the marine gastropod *Monoplex echo*, against mice [16]. Although the mechanism of action of Avt120 remains unclear, we found that GM1 and lysoGM1 strongly inhibited its ATP degradation and cytotoxic activity. They may have bound directly to an enzymatic domain of Avt120, or their binding may have protected the cell surface from its toxic effects. Avt120 cytotoxicity was magnesium and calcium dependent and reduced when these were chelated with EDTA. This effect is similar to that of an ATPase purified from sea urchin embryos [15].

The crude *A. villosa* toxin consisted of various proteins with different molecular weights. Avt120 was the most abundant, with the highest molecular weight. The presence of a toxin with ATP degradation activity is unique to *A. villosa*. Avt120 may act as phosphomonoesterase that hydrolyze free phosphate esters from ATP [21]. Therefore Avt120 is classified non-specific phosphatase at this stage. Further analysis is needed to decide this enzymatic activity. Besides Avt120, *A. villosa* contains actinoporin[18], which has cardiotoxic effects[3,17]. In rats, equinatoxin administration increased prostaglandin 2 production 8-fold [8]. Some protein toxins are indicated to trigger cytokine- or arachidonic acid-induced inflammation or fever [9], and others are thought to perform auxiliary functions for these activities.

Among the cell lines studied, Colo205 cells were most sensitive to Avt120. Avt120 receptor may be denser on Colo205 cells than in the other cell lines. Alternatively, Colo205 cells may have more sensitive response mechanisms to the ATP degradation activity of Avt120, resulting in greater cell death. When mice were inoculated intracerebrally, only a small amount of Avt120 induced death within minutes, indicating that Avt120 injured brain neuronal cells. Intramuscular Avt120 injection induced paralysis and muscle twitching, also suggesting that this toxin affected neurons.

Here, we demonstrated that Avt120 is a unique toxin with ATP degradation activity. Further investigations are necessary to reveal its biochemical and biological characteristics.

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References

1. Bernheimer AW, Avigad LS (1976) Proc Natl Acad Sci U S A 73; 467-471

2. Bressler JP, Belloni-Olivi L, Forman S (1994) Life Sci 54; 49-60

3. Bunc M, Drevensek G, Budihna M, Suput D (1999) Toxicon 37; 109-123

4. Chethankumar M, Srinivas L (2008) African Journal of Biochemistry Research 2; 8-14

5. Finney DJ (1971) 3rd Ed CambridgeUniversity Press, London

6. Hara-Yokoyama M, Hirabayashi Y, Irie F, Syuto B, Moriishi K, Sugiya H, Furuyama S (1995) J Biol Chem 270; 8115-8121

7. Hara-Yokoyama M, Kukimoto I, Nishina H, Kontani K, Hirabayashi Y, Irie F, Sugiya H, Furuyama S, Katada T (1996) J Biol Chem 271; 12951-12955

8. Ho CL, Ko JL, Lue HM, Lee CY, Ferlan I (1987) Toxicon 25; 659-664

9. Katayama T, Watanabe M, Tanaka H, Hino M, Miyakawa T, Ohki T, Ye LH, Xie C, Yoshiyama S, Nakamura A, Ishikawa R, Tanokura M, Oiwa K, Kohama K (2009) Am J Physiol Heart Circ Physiol 298; H505-514

10. Laemmli UK (1970) Nature 227; 680-685

11. Marino A, Valveri V, Muia C, Crupi R, Rizzo G, Musci G, La Spada G (2004) Comp

Biochem Physiol C Toxicol Pharmacol 139; 295-301

12. Mizuno M, Nozaki M, Morine N, Suzuki N, Nishikawa K, Morgan BP, Matsuo S (2007) Am J Pathol 171; 402-414

13. Nagai H, Oshiro N, Takuwa-Kuroda K, Iwanaga S, Nozaki M, Nakajima T (2002) Biosci Biotechnol Biochem 66; 2621-2625

14. Oshiro N, Iwanaga S, Nozaki M, Nakanose T, Uchida H (2001) Annual Report of Okinawa Prefectural Institute of Health and Environment 35; 133-136

15. Pratt MM, Otter T, Salmon ED (1980) J Cell Biol 86; 738-745

16. Shiomi K, Kawashima Y, Mizukami M, Nagashima Y (2002) Toxicon 40; 563-571

17. Suput D, Frangez R, Bunc M (2001) Toxicon 39; 1421-1427

18. Uechi G, Toma H, Arakawa T, Sato Y (2005) Toxicon 45; 761-766

19. Uechi G, Toma H, Arakawa T, Sato Y (2005) Protein Expr Purif 40; 379-384

20. Uechi G, Toma H, Arakawa T, Sato Y (2010) Toxicon 56; 1470-1476

21. Zhang L, Balcerzak M, Radisson J, Thouverey C, Pikula S, Azzar G, Buchet R (2005) The Journal of biological chemistry 280; 37289-37296

Figure legends

Figure 1. SDS-PAGE analysis of purified Avt120.

Lane M, molecular weight marker; Lane 1, crude toxin isolated from nematocysts; Lane 2, purified Avt120.

Figure 2. Nucleotide and deduced amino acid sequences of *A. villosa* Avt120 cDNA (DDBJ accession number AB576860).

The putative prepropeptide sequence is shaded, and the polyadenylation signal sequence is boxed. The deduced amino acid sequence is numbered starting from the presumed initiation methionine residue.

Figure 3. Multiple alignment of Avt120 with *Nematostella vectensis* homolog protein. (RefSeq accession number: XP_001636750, locus tag NEMVE_v1g240898). *N. vectensis* (RefSeq accession number: XP_001637427, locus tag NEMVE_v1g240466). Amino acid residues identical to Avt120 are shaded.

Figure 4. Mg, Ca, and EDTA affecting against cytotoxic activity of Avt120.

Colo205 cells (1×10^5 cells/100 µl) were incubated with 38.4 ng/ml Avt120 in the presence of 10 µl PBS, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, or 3 mM EDTA (pH 8.0).

Figure 5. ATP degradation inhibition by pre-incubation with gangliosides.

ATP degradation inhibition after 1 h pre-incubation of 5 µg Avt120 with 1 µM GM1, GM2, GM3, GD1a, GD1b, GD2, GT1b, lysoGM1, Asialo GM1, Asialo GM2,

ganglioside mixture, sucrose, glucose, fructose, phosphatidylcholine, phosphatidylserine, sphingomyelin, cholesterol, linoleic acid, arachidonic acid or 0.5% TritonX-100. Relative inhibition activity were determined as compared with inhibitor-untreated controls.

Figure 6. Cytotoxicity inhibition against Colo205 cell by gangliosides.

Colo205 cell death after pre-incubation with 1 μM GM1 or 1 μM lysoGM1 was monitored by MTT assay. Relative O.D. reduction were determined as compared with inhibitor-untreated controls.

Figure 1

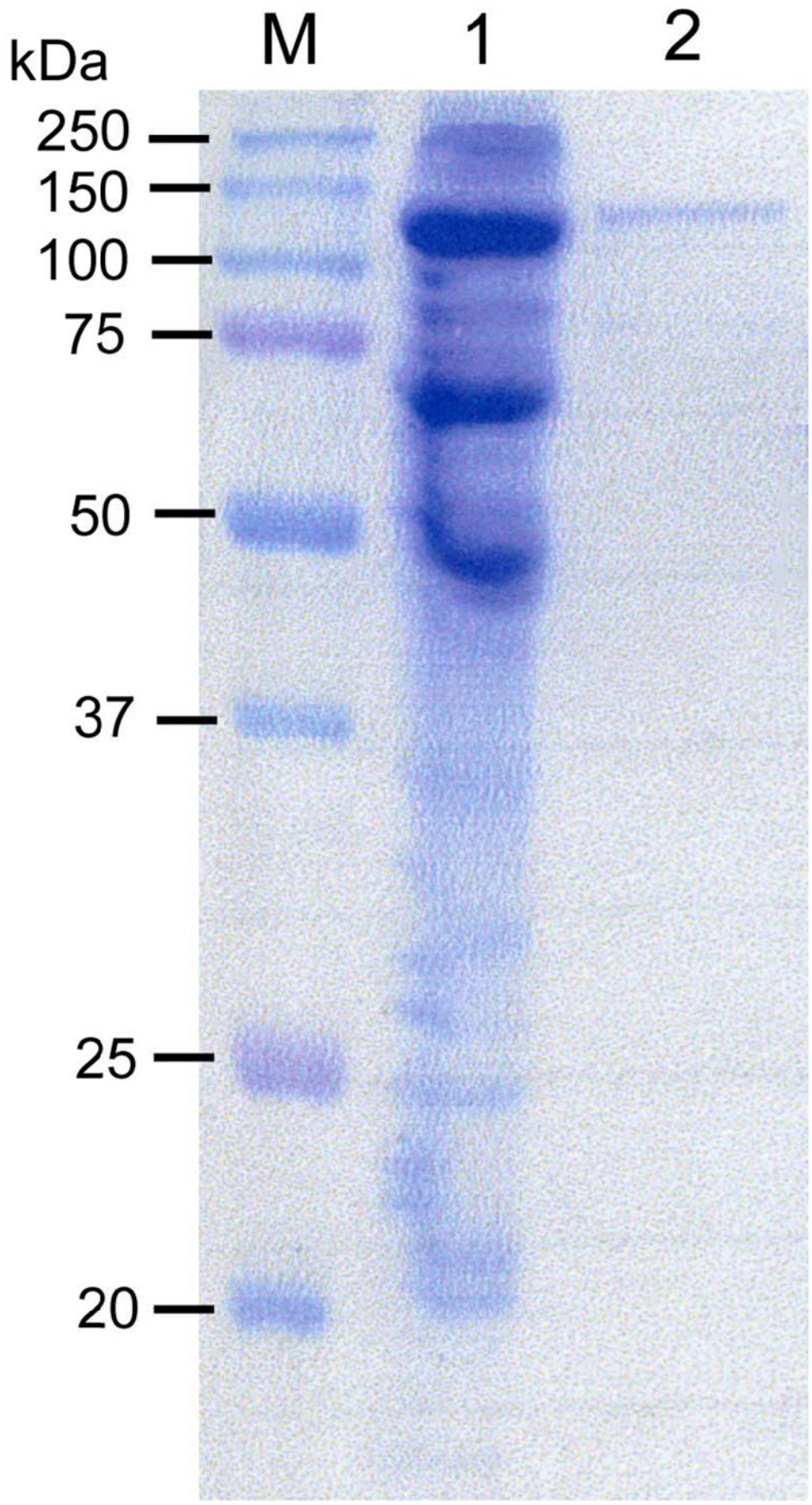

Figure 2

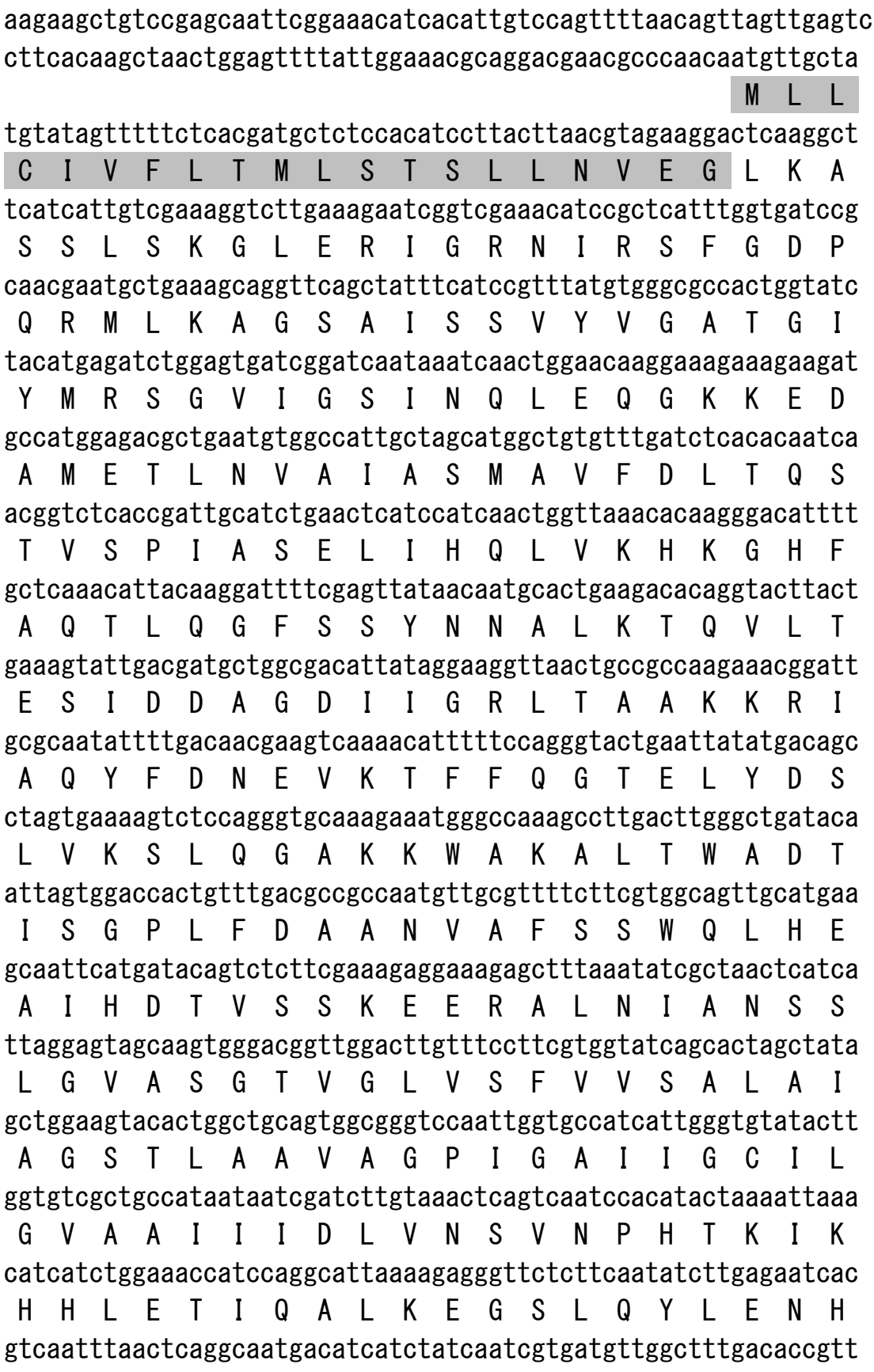
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Figure 2

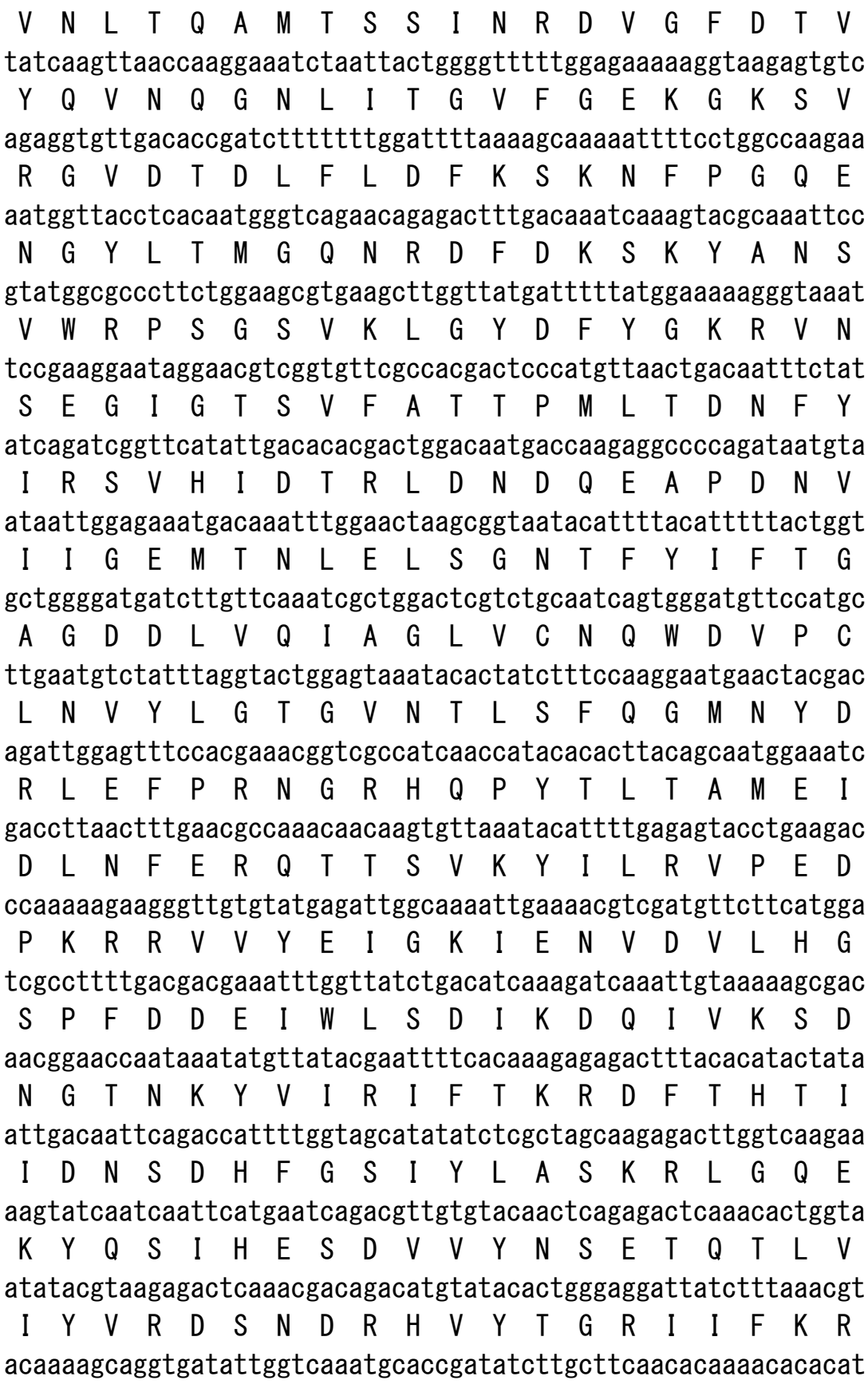
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Figure 2

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Figure 2

gtcagaggaaaaaaaaaaaaaaaaaaaaaa

Figure 3

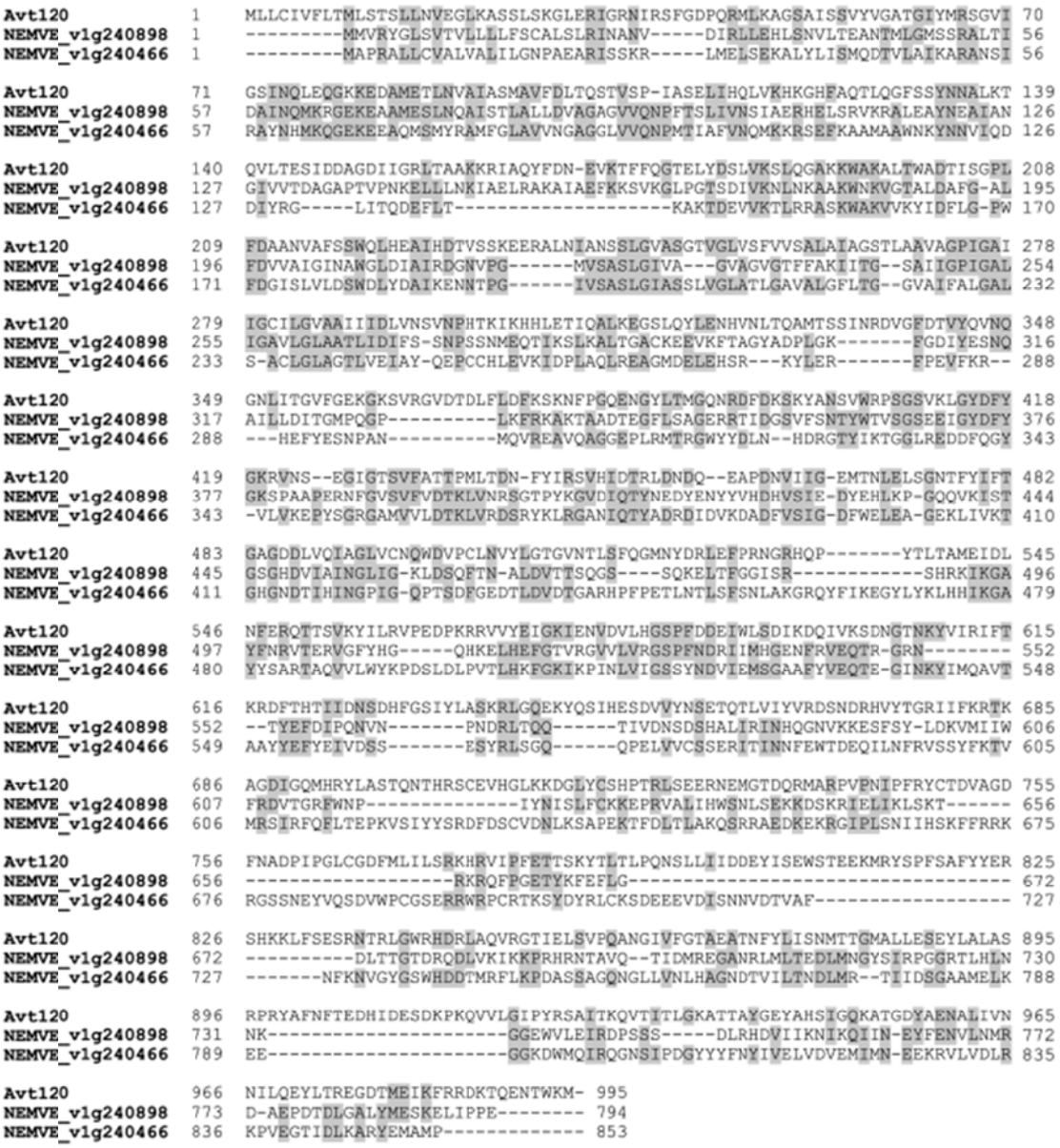
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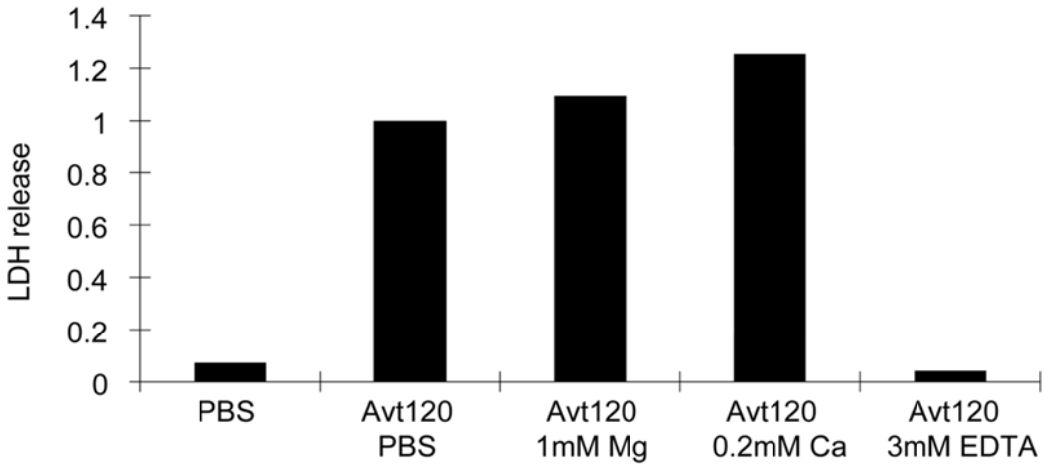
Figure 4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1.2
1
0.8
0.6
0.4
0.2
0
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Avt120 PBS
Avt120 1mM Mg
Avt120 0.2mM Ca
Avt120 3mM EDTA

Figure 5

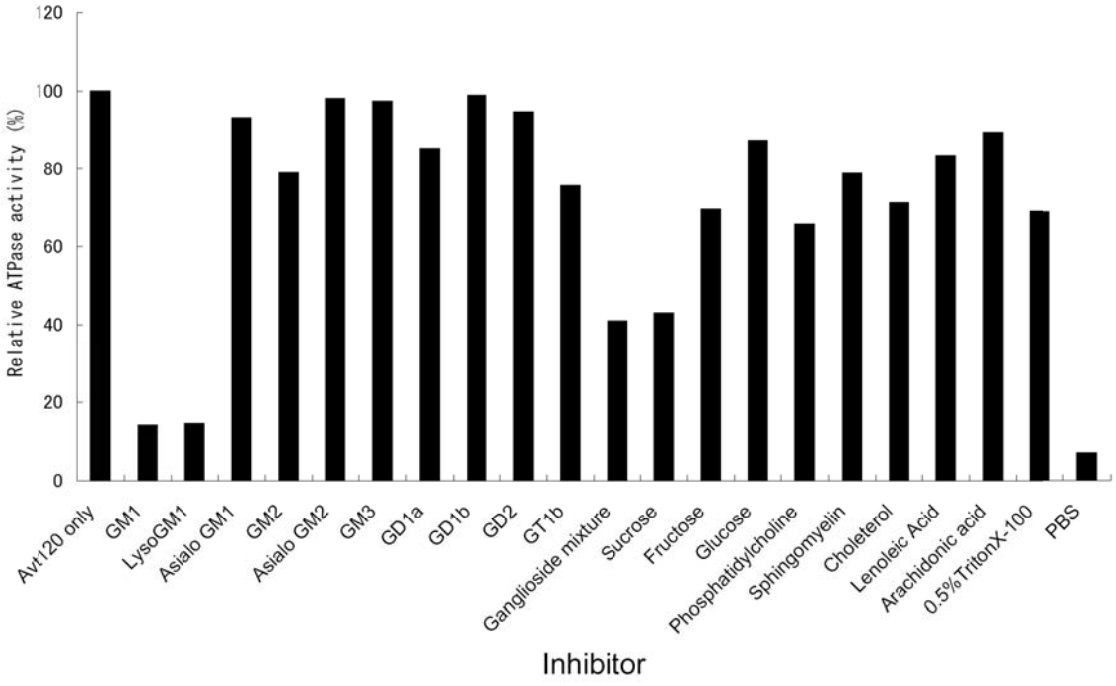
Relative ATPase activity (%)
120
100
80
60
40
20
0
Avr120 only
GM1
LysoGM1
Asialo GM1
GM2
Asialo GM2
GM3
GD1a
GD1b
GD2
GT1b
Ganglioside mixture
Sucrose
Fructose
Glucose
Phosphatidylcholine
Sphingomyelin
Choleterol
Lenoleic Acid
Arachidonic acid
0.5%TritonX-100
PBS
Inhibitor

Figure 6

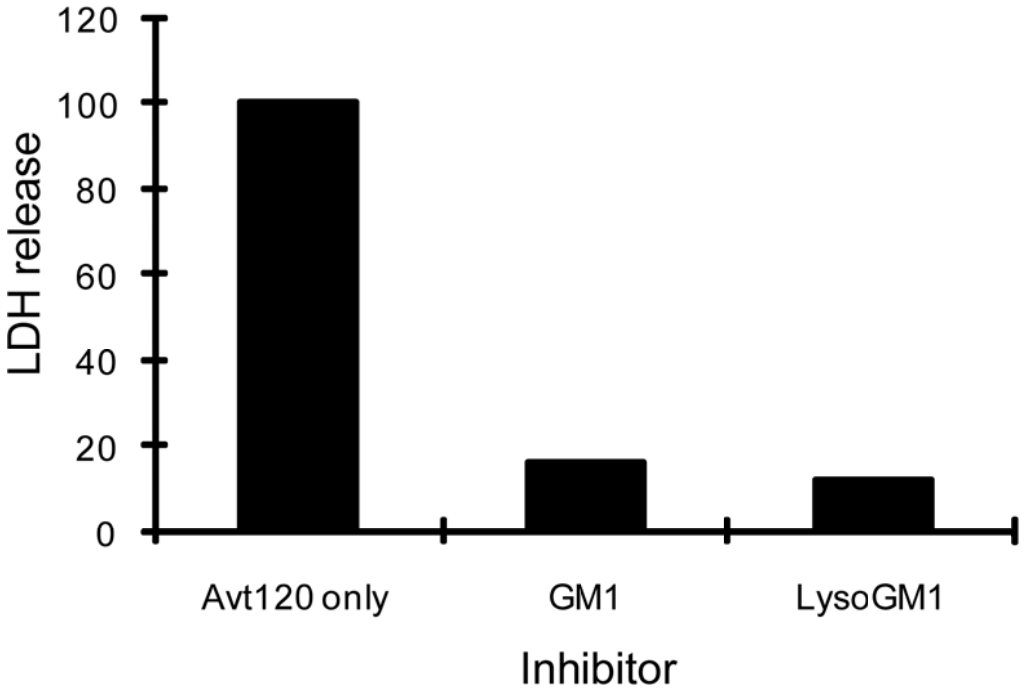
120
100
80
60
40
20
0
LDH release
Avt120 only
GM1
LysoGM1
Inhibitor

Table 1. Column purification of Avt120 protein.

Fraction	Protein (mg)[a]	Total activity µmol/h [b]	Specific activity µmol/h/mg	Purification (fold)	Yield (%)
Total extracted protein	20.0	121.8	6.1	1.0	100.0
CM-Sephadex	12.6	85.6	6.8	1.1	63.0
CM-5PW	6.3	45.1	7.2	1.2	31.5
Superdex75HR	1.2	12.0	10.0	1.6	6.0

[a]Total protein concentration was measured by BCA method

[b]ATP degradation assay was performed by incubation with 5µg of purified Avt120 and 1 mM ATP at 37°C for 1h. Degraded ATP was monitored by reverse phase HPLC.

Table 2. Mouse LD_{50} determination of purified Avt120 toxin

Avt120 (ng/mouse)	Survived
200	0/4
100	2/4
50	3/4
0	4/4

One hundred micro liter of each toxin dilution (200, 100, 50 and 0 ng) was intravenously injected against each 4 mice. Total sixteen ICR mice (20–25 g) were observed after 24 h and LD_{50} value was determined by probit method [5].