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Molybdophyllysin, a toxic metalloendopeptidase from the tropical toadstool, *Chlorophyllum molybdites*

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ABSTRACT

A toxic protein, dubbed molybdophyllysin, was isolated from the tropical toadstool *Chlorophyllum molybdites* by following its lethal effect in mice. Analysis of the protein using SDS–PAGE revealed a single 23-kDa band. Sequence analysis of molybdophyllysin tryptic fragments showed that this protein is highly homologous to metalloendopeptidases (MEPs) obtained from edible mushrooms, such as *Grifola frondosa*, *Pleurotus ostreatus*, and *Armillaria mellea*. These proteins include a HEXXH+D zinc-binding motif known as aspzincin. Accordingly, molybdophyllysin is a member of the deuterolysin family of zinc proteases. Molybdophyllysin retained its proteolytic activity at temperatures up to 60 °C with an optimum pH of 7.0. The activity was inhibited by both 1,10-phenanthroline and *N*-bromosuccinimide, but molybdophyllysin exhibited strong resistance to SDS.

1. Introduction

Mushroom toxins have been extensively studied because of their remarkable physiological properties. A variety of toxins have been characterized to date, some of which have become useful in biomedical research.[1,2] Clarification of the chemical structures and biological properties of mushroom toxins would pave the way for detoxification and treatment of mushroom poisoning.[1,3]

The agaricaceous toadstool *Chlorophyllum molybdites* (formerly *Lepiota morganii*) is common in tropical and subtropical regions. Poisoning resulting from ingestion of *C. molybdites* toxin has been known for the past 100 years, ever since the range of the fungus extended from the southern to middle countries in America.[4] In Japan, which is in the temperate zone, *C. molybdites* was first recognized in 1937, and prior to the 1970s, there were few reported cases of poisoning due to this fungus. In recent years, however, *C. molybdites* has become widely distributed in Japan, probably due to global climate change, and this has led to an increase in cases of mushroom poisoning.[5] Although, the poisoning caused by *C. molybdites* is rarely fatal, the symptoms are severe, and include vomiting, diarrhea, chills, intestinal pain, stomach ache, gastrointestinal hemorrhage, and in some cases convulsions.[6–8]

Previous studies on the toxic components of *C. molybdites* suggested the presence of a cholinergic compound.[9,10] However, Eilers and Nelson later demonstrated that the toxic component is proteinaceous, and they reported partial purification and characterization of a toxic protein.[4] In addition to the protein toxin, nontoxic alkaloids,[11] steroids,[12] and lectins[13] have been isolated from *C. molybdites*.

The recent increase in the incidence of *C. molybdites* poisoning in Japan prompted us to reinvestigate the toxic components produced by this fungus. Our research was guided by following the lethal effect of *C. molybdites* components in mice, and resulted in the isolation of a toxic protein, dubbed molybdophyllysin. We found that molybdophyllysin is highly homologous to various metalloendopeptidases (MEPs) previously identified in edible mushrooms, such as *Grifola frondosa*, *Pleurotus ostreatus*,[14] and *Armillaria mellea*.[15] Here, we report the isolation of molybdophyllysin, its partial amino acid sequence, and its enzymatic properties.

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2. Results and discussion

2.1. Isolation

In order to isolate the toxic component, mice were intraperitoneally injected with extracts of *C. molybdites* fruit bodies according to the method of Eilers and Nelson.[4] Frozen *C. molybdites* fruit bodies were independently extracted with MeOH, H_2O, MeCN–H_2O (1:1), and MeCN–H_2O (1:1) containing 1% TFA. Only the water extract was lethal to mice, and its toxicity was lost after heating the extract for 20 min at 100 °C. When the water extract was dialyzed (MW 12,000–14,000) against water, the toxicity was associated with the non-dialyzate. These preliminary experiments suggested that the toxin is a proteinaceous compound. The effect of pH on the toxicity of the water-soluble component was also crudely examined. When aliquots of the water extract were adjusted to pH 4.0–11.0 and left to stand overnight at 4 °C, all the resulting fractions were toxic to mice.

Purification of the responsible proteinaceous toxin was complicated by contamination of the extract with colored polyphenols, which prevent UV quantification of the protein and may introduce artifacts into the bioassay.[4] In general, agaricaceous mushrooms produce an abundance of colored polyphenols. As *C. molybdites* contains many polyphenol precursors in its fruiting body, a large amount of colored polyphenols were produced by air oxidation during extraction in our experiments, and their removal was difficult. Accordingly, the extraction was performed in the presence of the reducing agents phenylthiourea and sodium dithionite to prevent formation of the polymers as far as possible. The resulting extract was evaporated in vacuo and the remaining residue was dialyzed against water containing the reducing agents, and the non-dialyzate was then lyophilized. The crude material was chromatographed on a cation exchange resin (CM-52, pH 4.5 in 0.02 M citrate–NaOH buffer) with stepwise elution with NaCl, resulting in complete removal of the residual colored materials. The cation exchange fraction eluted with 0.10 M NaCl was successively chromatographed on an anion exchange resin (DE-52, pH 7.2 in 0.05 M Tris–HCl buffer) with stepwise elution with NaCl, and then by gel filtration (Sephadex G-50 at pH 6.8 in 0.05 M Tris–HCl buffer) with monitoring of the eluate by UV absorbance (280 nm). In the gel filtration chromatogram, fractions corresponding to the major peak were toxic to mice, and these fractions were combined and concentrated using ultrafiltration. After this manipulation, the toxicity of the protein component decreased, even if the sample solution was kept in a refrigerator overnight. The cause of the decrease in toxicity was assumed to be the result of a conformational change in the protein. Conformational changes often occur when proteins are left in a dilute solution because the degree of hydration is increased, which leads to weakening of the hydrogen bonds that serve as the major force maintaining protein conformation. In order to retain toxicity (i.e., to retain the original hydrogen bonding), glycerol was added to the sample after ultrafiltration. Addition of glycerol and storage of samples in a refrigerator resulted in retention of toxicity for over one month.

Further purification of the protein using anion exchange fast protein liquid chromatography (FPLC; Mono Q, pH 7.2 in 0.05 M Tris–HCl buffer) also caused a loss of toxicity. Accordingly, the FPLC separation was performed by addition of glycerol to the eluent and gradient elution with NaCl monitored by UV. The loss of toxicity was again overcome (Fig. 1). The main peak was collected and concentrated using ultrafiltration in the presence of glycerol. Analysis of the fraction using SDS–PAGE showed a single band at about 23 kDa (Fig. 2). The protein exhibited toxicity and was also found to have proteolytic activity (vide infra). Accordingly, we called the toxic protein molybdophyllysin.

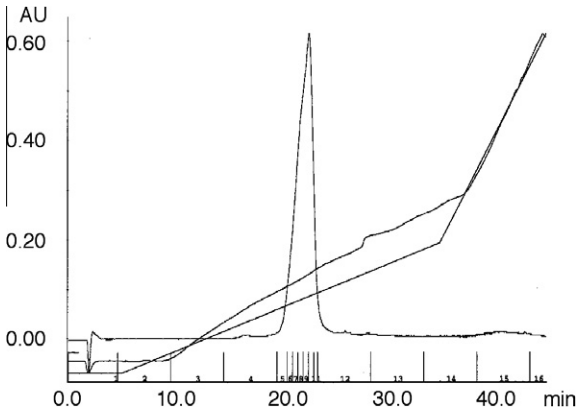

Figure 1. Purification of molybdophyllysin using FPLC. Conditions: Mono Q HR 5/5 column; elution with a gradient of NaCl (0.0–0.5 M) in 0.05 M Tris–HCl buffer (pH 7.2) containing 0.2% glycerol; flow rate: 0.5 ml/min; detection at 280 nm.

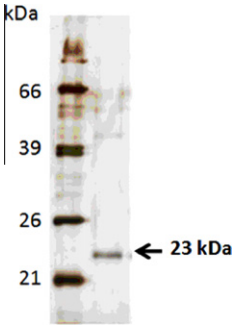

Figure 2. SDS–PAGE analysis of molybdophyllysin. The left lane contains molecular weight markers. The right lane contains molybdophyllysin isolated using FPLC.

2.2. Amino acid sequence analysis

The molybdophyllysin sample applied to SDS–PAGE was blotted to a PVDF membrane and then analyzed using a protein sequencer. The N-terminal amino acid sequence of molybdophyllysin was determined as: ETYVGXSTSQKSALTTAAPNA. In order to clarify the identity of the amino acid residue denoted as X, sugar analysis was performed on the protein using both enzymatic and chemical degradation. Molybdophyllysin was deglycosylated with endoglycosidase H and the resulting products were analyzed using SDS–PAGE, which indicated no change in the molecular mass of the protein. Further, the chemical degradation test using a PAS-staining was also negative for the presence of sugar. These results indicated that amino acid residue number 6 (X) is likely cysteine or cystine, leading to a final N-terminal sequence of ETYVGCSTSQKSALTTAAPNA.

In order to obtain additional sequence information for molybdophyllysin, the protein was digested with trypsin and the resulting peptide fragments were separated using reversed-phase HPLC and then sequenced. Approximately twelve peptide peaks were obtained upon tryptic digestion of reductive pyridylethylated molybdophyllysin. The chromatographic elution profile of the tryptic digest is shown in Figure 3. The amino acid sequences of the peptides corresponding to 4 of the 12 peaks, P-2, P-5, P-10, and P-11, were elucidated. The 17 N-terminal amino acids of peaks P-10 and P-11 were identical.

2.3. Homology with other known MEPs

The homology of the N-terminal peptide of molybdophyllysin and the fragment peptides corresponding to HPLC peaks P-2, P-5,

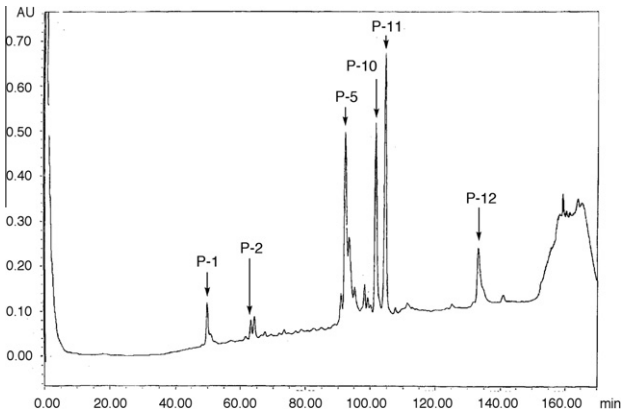

Figure 3. HPLC separation of molybdophyllysin tryptic digestion products. Conditions: Grand Pak Wakosil II 5C18RS column; eluent A: 1% MeCN-0.1% TFA and eluent B: 90% MeCN-0.1% TFA, gradient: 0–10 min, 0% B; 10–136 min, 0–42% B; 136–145 min, 42–100% B; 145–155 min, 100% B; 155–160 min, 0–100% B; flow rate: 80 μl/min; detection at 215 nm.

and P-10 with other known proteins was examined using the MEROPS database (Fig. 4). The homology search suggested that molybdophyllysin is homologous to MEPs, including GFMEP, POMEP, and AMMEP, isolated from the basidiomycete fungi *Grifola frondosa*, *Pleurotus ostreatus*, and *Armillaria mellea*, respectively.[14,15] The 21 N-terminal amino acid residues of molybdophyllysin showed 43-65% identity with residues 1-21 of MEPs isolated from *G. frondosa*, *P. ostreatus*, and *A. mellea*. The amino acid sequences of the molybdophyllysin peptides corresponding to HPLC peaks P-2, P-5, and P-10 demonstrated 58-84% identity with peptides derived from the three other species of fungi (Fig. 4).

The sequence of molybdophyllysin was also quite similar to other known MEPs, including deuterolysin,[16] penicillolysin,[17] and MEPs from *Aspergillus flavus*[18] and *Aspergillus fumigatus*.[19] These MEPs contain an aspzincin zinc-binding motif, defined by the 'HEXXH+D' sequence.[20] This motif was also found in the sequence of the peptide fragment corresponding to HPLC peak P-5, suggesting that molybdophyllysin should be classified as a member of the deuterolysin family of MEPs (peptidase family M35).

2.4. Enzymatic properties of molybdophyllysin

Since the homology search results suggested that molybdophyllysin is a MEP, its enzymatic properties were examined using azocasein as a substrate. The optimum pH for molybdophyllysin proteolytic activity was 7.0, within the range of pH 4.0–11.0 (Fig. 5). Interestingly, the activity was buffer-dependent; that is, the activity decreased by 40% when the analysis was conducted in 0.1 M Tris–HCl buffer instead of sodium borate buffer at pH 8.0. The thermostability of molybdophyllysin's proteolytic activity was also examined using azocasein as a substrate. Proteolytic activity was maintained to at least 60 °C at pH 6.5 (Fig. 6). The effect of pH on the proteolytic activity was examined by pre-incubating molybdophyllysin for 10 h at various pH levels across the range 2.0–11.0 prior to examining the proteolytic activity (Fig. 7).

We also examined the effect of various compounds on the proteolytic activity of molybdophyllysin in order to more fully characterize its enzymatic properties (Table 1). The proteolytic activity of molybdophyllysin was partially inhibited in the presence of several divalent metals, including Cu^{2+}, Cd^{2+}, and Hg^{2+} (1 mM each). In contrast, Mg^{2+}, Mn^{2+}, Ca^{2+}, and Zn^{2+} (1 mM each) had no effect on the activity. Proteolytic activity was reduced in the presence of metal chelators such as 1,10-phenanthroline and EDTA, as well as in the presence of the reducing agent 2-mercaptoethanol and PCMB (*p*-chloromercuribenzoic acid), a specific inhibitor of thiol-containing enzymes. The activity was completely inhibited by *N*-bromosuccinimide (1 mM), which is known to modify tryptophan residues. On the other hand, PMSF (phenylmethylsulfonyl fluoride, a specific inhibitor of serine proteases), pepstatin A (a specific inhibitor of acidic proteases), phosphoramidon (a specific inhibitor of neutral endopeptidases (NEPs)), 8-hydroxyquinoline (a metal chelator), and iodoacetamide (a specific inhibitor of thiol-containing enzymes) had no effect. Molybdophyllysin also exhibited a strong resistance to SDS.

3. Conclusions

In this study, we identified and characterized a toxic component isolated from the mushroom *C. molybdites*. Guided by its lethality in mice, we isolated a toxic protein with a molecular mass of 23 kDa that we termed molybdophyllysin. Our findings stand in contrast to those of Eilers and Nelson, who reported that the toxic component of this fungus is a polymeric protein with a molecular mass in excess of 400 kDa, comprised of monomers of molecular mass 40–60 kDa.[4] Molybdophyllysin is a relatively small protein; accordingly, it may correspond to the monomer reported by Eilers and Nelson.

Molybdophyllysin was found to have proteolytic activity, which was presumed from analysis of its partial amino acid sequence. The sequence data also suggested that molybdophyllysin is a member of the deuterolysin family of MEPs, containing an aspzincin zinc-binding motif.

Homology searching indicated that molybdophyllysin is highly homologous to several MEPs, including GFMEP, POMEP, and AMMEP. Metalloendopeptidases are found in many fungi and bacteria; however, no toxicity has been associated with the bacterial and

	N-terminal		Peak 2	
molybdophyllysin	ETYVGCSTS-QKSALTTAAPNA	21	HSTVLSHFT	
GFMEP	TYNGCSSSEQ-SALAAAASAA	20	HSTVLQHYT	61
POMEP	ATFVGCSATRQ-TQLNAAASQA	21	YNTVLSHFS	62
AMMEP	ISYNGCTSSRQ-TTLVSAAAAA	21	HSTVLSCFS	62
	☆☆ ☆ ☆ ☆☆ ☆		☆☆☆	

	Peak 5		Peak 10	
molybdophyllysin	AGTLIHESSHFIKNGGT		SLAKSSPAQAITNADSHEY	
GFMEP	AGTLVHESSHFTRNGGT	128	SLATMDPDKAVMNADNHEY	156
POMEP	GGTLIHESSHFTRNGGT	129	SLARSNPAQAIDNADSHEY	159
AMMEP	GGTLIHESSHFTIICGT	129	SLASSNPSEAIKNADNYEY	157
	☆☆☆ ▲▲☆☆▲☆ ☆☆		☆☆☆ ☆ ☆ ☆☆☆ ☆☆	

Figure 4. Homology of molybdophyllysin with other known metalloendopeptidases. Residues common to the four metalloendopeptidases are indicated by open asterisks. Closed triangles show the zinc-binding motif. These MEPs were derived from the following fungi: Molybdophyllysin, *Chlorophyllum molybdites*; GFMEP, *Grifola frondosa*; POMEP, *Pleurotus ostreatus*; AMMEP, *Armillaria mellea*.

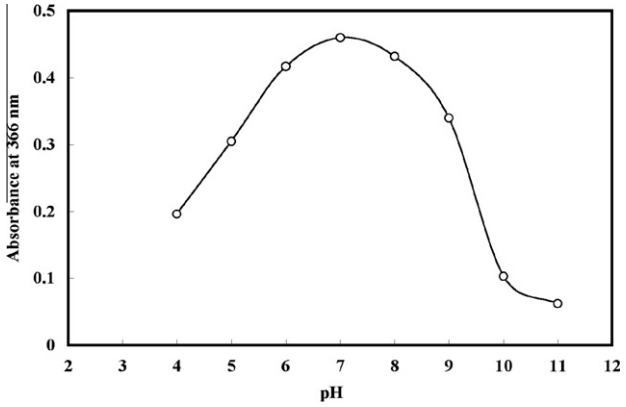

Figure 5. Effect of pH on the proteolytic activity of molybdophyllysin. The enzyme activity was measured as described in the Section 4 using the following buffers: 0.05 M acetate buffer (pH 4 and 5), 0.05 M phosphate buffer (pH 6 and 7), 0.05 M borate buffer (pH 8 and 9), 0.05 M glycine–NaOH buffer (pH 10 and 11).

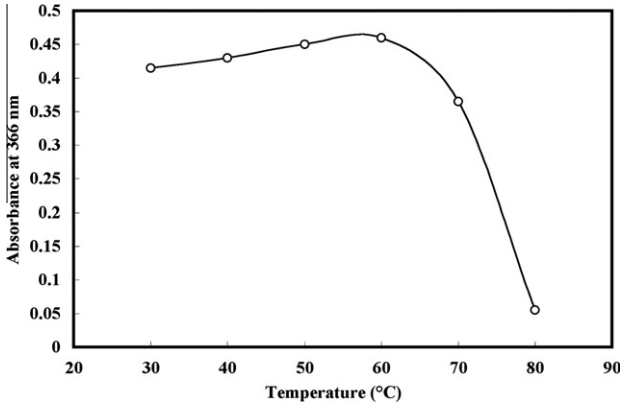

Figure 6. Thermostability of molybdophyllysin proteolytic activity. Molybdophyllysin (2 μg) in 100 μl of 0.05 M phosphate buffer (pH 6.5) containing 10% glycerol was incubated at various temperatures for 10 min. The residual proteolytic activity was then measured as described in the Section 4.

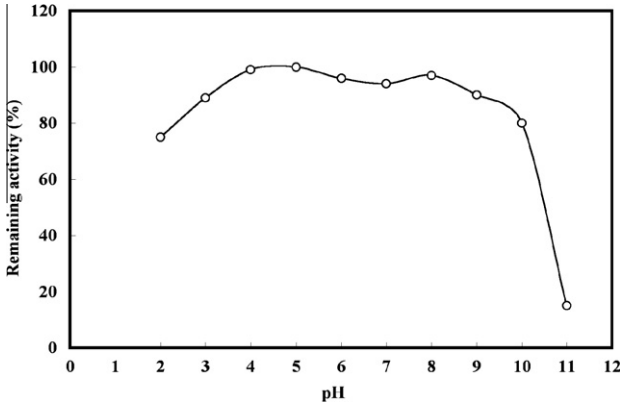

Figure 7. The pH stability of molybdophyllysin proteolytic activity. The enzymatic activity of molybdophyllysin was assayed after pre-incubation in buffers at various pH levels for 10 h at 30 °C. Buffer: 0.05 M glycine–HCl (pH 2 and 3), 0.05 M acetate buffer (pH 4 and 5), 0.05 M phosphate buffer (pH 6 and 7), 0.05 M borate buffer (pH 8 and 9), 0.05 M glycine–NaOH buffer (pH 10 and 11).

fungal MEPs thus far described. In this respect, molybdophyllysin is unique and it is of great interest to clarify the relationship between its toxicity and its MEP activity. Toward this end, determinations of the full sequence and three-dimensional structure of molybdophyllysin are now underway.

Table 1
Effect of various compounds on the proteolytic activity of molybdophyllysin

Compounds	Concentration	Remaining activity (%)
Divalent metal chlorides		
Mg, Mn, Ca, Zn	1 mM	95–103
Cu	1 mM	63
Hg, Cd	1 mM	20–35
Protease inihibitors		
PMSF[a]	1 mM	99
Pepstatin A	0.2 mM	98
Phosphoramidon	0.2 mM	104
1,10-Phenanthroline	1 mM	20
EDTA	1 mM	78
8-Hydroxyquinoline	1 mM	107
Other reagents		
Iodoacetamide	1 mM	105
N-Bromosuccinimide	1 mM	0
PCMB[b]	1 mM	53
SDS	1%	115
2-Mercaptoethanol	1%	29

[a] PMSF: phenylmethylsulfonyl fluoride.
[b] PCMB: *p*-chloromercuribenzoic acid.

4. Experimental

4.1. Isolation of a toxic protein, molybdophyllysin, from Chlorophyllum molybdites

The fruit bodies of *C. molybdites* were collected during 1995–2001 in Kochi and Oita prefectures, Japan and stored in −30 °C freezer. The whole fruit bodies (1.0 kg) were cut into pieces and soaked in water (2.0 l) containing reducing agents, phenylthiourea (2.0×10^{-3} M) and sodium dithionite (2.0×10^{-4} M), at room temperature (rt) overnight. The mixture was filtered through gauze and the filtrate was concentrated in vacuo approximately to a one thirds of the initial volume and then it was dialyzed (MW 12,000–14,000) against water (6.0 l) containing phenylthiourea (2.0×10^{-3} M) and sodium dithionite (2.0×10^{-4} M) at rt overnight. The resulting non-dialyzate was subjected to centrifugation and the brown insoluble precipitates produced were removed. Then the supernatant was concentrated approximately to the half volume. Until no further brown polymers were produced, the manipulation (centrifugation and concentration) was repeated. The concentrated solution was dialyzed (MW 12,000–14,000) against water and the resulting non-dialyzate was lyophylized to give a crude extract (1.77 g). The second extraction in a similar manner gave an extract (0.23 g). The combined extracts (2.0 g) were applied to the next cation exchange chromatography.

A cation exchange resin (CM-52, Whatman) was swelled with 0.02 M citrate–NaOH buffer (pH 4.5). The extract (1.0 g) was chromatographed on the resin (300 ml) by stepwise elution with the following solutions; (a) buffer (300 ml), (b) buffer (900 ml), (c) 0.05 M NaCl in the buffer (900 ml), and (d) 0.10 M NaCl in the buffer (900 ml). Thus obtained fractions were concentrated, in order to adjust the concentration to be 0.5 M with regard to NaCl. Each of the concentrated fractions was dialyzed (MW 12,000–14,000) against water (900 ml) at rt overnight and then the non-dialyzate was lyophilized. The yields were (a) 135 mg, (b) 58 mg, (c) 107 mg, and (d) 64 mg, respectively. The lethal effect on mice was observed in the fraction d by injection of 2.5 mg/capita. The active fraction was next applied to an anion exchange chromatography.

An anion exchange resin (DE-52, Whatman) was swelled with 0.05 M Tris–HCl buffer (pH 7.2). The above fraction d (61 mg) was chromatographed on the resin (143 ml) by stepwise elution with the following solutions; (a) buffer (429 ml), (b) 0.05 M NaCl in the buffer (429 ml), (c) 0.10 M NaCl in the buffer (429 ml), (d) 0.15 M NaCl in the buffer (429 ml), and (e) 0.20 M NaCl in the buffer (429 ml). The fractions (143 ml each) were concentrated, in order to adjust the concentration to be 0.5 M with regard to NaCl. Each of the solutions was dialyzed (MW 12,000–14,000) against water (900 ml) at rt overnight and then the non-dialyzate was lyophilized. The yields were (a) 41 mg, (b) 42 mg, (c) 30 mg, (d) 44 mg, and (e) 27 mg, respectively. The lethal activity was observed in the fraction d by injection of 2.5 mg/capita. The fraction d was further purified by a gel filtration.

Sephadex G-50 (GE Healthcare, UK) was swelled with 0.05 M Tris–HCl buffer (pH 6.8). The above fraction d (5.2 mg) was chromatographed on the gel (95 ml). Using the same buffer as the eluate, the 5 ml each was fractionated to twenty fractions. Guided by the UV absorbance (280 nm), the fractions No 7-8 and 11-13 were respectively combined and concentrated to 1.0 ml by ultrafiltration (Centriprep YM-10 (MW 10,000), Millipore, 20,000×*g*, 4 °C). To these solutions were added glycerol (0.25 mL, 20% of the solution) in order to keep the activity. The aqueous glycerol solution was also used for the bioassay. The total amount of thus obtained solutions were injected to one mouse, respectively. The activity was observed in the combined fraction of No 11-13. Accordingly, this fraction (corresponding amount of 5.6 mg after the anion exchange chromatography) containing glycerol was applied to FPLC using Mono Q HR 5/5 column (anion exchange resin, GE Healthcare, UK). Elution was conducted with gradient NaCl (0.0–0.5 M) in 0.05 M Tris–HCl buffer (pH 7.2) containing 0.2 % glycerol (flow rate: 0.5 ml/min, UV detection at 280 nm). To the second fraction (rt 20.0–25.0 min) was added glycerol (20% of the solution) and the mixture was concentrated by Ultrafree (10,000 MWCO, Millipore, Bedford) at 4 °C, 7000×*g* to 500 μl. The total protein content was about 605 μg estimated from Bradford method.[21] The lethal effect was observed in the concentrated fraction by injection of the whole amount to one mouse. Finally, the LD_{100} value was about 0.1 mg/capita when molybdophyllysin was injected intraperitoneally to a mouse.

4.2. Bioassay on mice

The extract was dissolved into saline (0.3 ml) and the mixture was injected intraperitoneally to a mouse (clean ddY strain, female, weight: 25–30 g). When the lethal effect was observed within 3 h, the sample was regarded as toxic. Enormous bleeding in the intraperitoneal cavity of the dead mice was observed.

4.3. Sodium dodecyl sulfate SDS–PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

SDS–PAGE (12.5% polyacrylamide gel) was performed according to the method of Laemmli.[22] Proteins in the gel were detected by silver staining. A calibration kit (Amresco, Ohio, USA) was used as the standard molecular mass markers.

4.4. Sugar chains analysis of molybdophyllysin

Sugar chains analysis of molybdophyllysin was performed by two methods, enzymatic and chemical methods. The first method, deglycosylation with endoglycosidase H (Biolabs Inc. New England), was as follows: After denatured with SDS, molybdophyllysin (20 μg) in 0.05 M Tris–HCl buffer (20 μl, pH 7.5) was incubated with the enzyme (5 μl) for 24 h at 30 °C. After lyophilization, the reaction mixture was analyzed by SDS–PAGE. The second method, chemical deglycosylation, was performed with G.P. SENSOR (Seikagaku Corp.) using the PVDF membrane blotting protein after SDS–PAGE.

4.5. Reductive pyridylethylation of molybdophyllysin

Molybdophyllysin (about 90 μg) was dissolved in 0.5 M Tris–HCl buffer (100 μl, pH 8.5) containing DTT (1 mg) and EDTA (10 mM). After replacement of air in the tube with nitrogen, the reaction mixture was stood for 2 h at rt. Then, 4-vinylpyridine (2 μl) was added, and the reaction mixture was incubated for 4 h in the dark at rt. After the incubation, the reaction mixture was dialyzed (MW 12,000–14,000) against water to exclude reagents, and the non-dialyzate was lyophilized.

4.6. Trypsin digestions of reductive pyridylethylated molybdophyllysin

The pyridylethylated molybdophyllysin (10 μg) was dissolved in 8 M urea solution (1 μl) containing NH_4HCO_3 (50 mM) and denatured for 2 h at 37 °C. After the denaturation, seven times volume of NH_4HCO_3 solution (50 mM) and 1/50 (enzyme/substrate) of modified trypsin (Promega, USA) were added to the reaction mixture. The mixture was incubated for 4 h at 37 °C, and then the mixture was lyophilized.

4.7. Reverse phase HPLC of tryptic digests of reductive pyridylethylated molybdophyllysin

The tryptic digests of pyridylethylated toxic component were separated by reversed-phase HPLC (Waters 600 pump, 486 Tunable absorbance detector, USA), Grand Pak Wakosil II 5C18RS (ID 1 × 150 mm column, Wako Chemicals). The tryptic digests were dissolved in water (5 μg/μl) and 20 μl of the solution was used at one injection. The HPLC system was operated at flow rate of 80 μl/min, detection at 215 nm. Elution was conducted with two solvents, eluent A: 1% MeCN-0.1% TFA and eluent B: 90% MeCN-0.1% TFA, and these were mixed as following gradient: 0–10 min, 0% B; 10–136 min, 0–42% B; 136–145 min, 42–100% B; 145–155 min, 100% B; 155–160 min, 100–0% B.

4.8. Amino acid sequence analysis

The N-terminal amino acid sequences of molybdophyllysin and its tryptic digests separated by reversed-phase HPLC were analyzed with protein sequencer PPSQ-10 (Shimadzu) at the Gene Research Center of Hirosaki University.

4.9. Proteolytic activity

Proteolytic activity of molybdophyllysin was tested on azocasein (Sigma, USA) as a substrate.[15] Aliquots were added to 2 ml of azocasein (3 mg/ml) in 0.05 M phosphate buffer, pH 7.0 containing 10% glycerol and incubated at 37 °C for 30 min to 1 h. To the mixture was added 12% (w/v) trichloroacetic acid (1 ml) in order to precipitate the undegraded azocasein. After 20 min, the solution was centrifuged and the absorbance at 366 nm of the supernatant was measured.

4.10. Effects of pH on enzyme activity

The effects of pH on the proteolytic activity of molybdophyllysin were also checked using azocasein as a substrate by the method described above (Section 4.9). To measure the pH stability, the enzyme solutions were preincubated at various pHs (pH 2–11) at 30 °C for 10 h, and the remaining activity was measured after adjusting to pH 7.0.

4.11. Effects of temperature on stability

The thermostability of molybdophyllysin was also checked as the same manner as described above (Section 4.9). After heating the each enzyme solution (pH 6.5) for 10 min at various temperature from 30 to 70 °C, the remaining proteolytic activity was measured.

4.12. Effects of additives on proteolytic activity

The effects of various compounds on proteolytic activity of molybdophyllysin were also checked as the same manner as described above (Section 4.9). Molybdophyllysin was mixed with each reagent, incubated at 30 °C for 30 min prior to the addition of the substrate, and then the remaining activity was measured.

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