Brief Report

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# Structural analyses of a hemolytic compound found in an extract of *Hypsizygus marmoreus* fruiting bodies at a low pH

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**Summary** **The current study determined the structure of a hemolytic compound found in an extract from the fruiting bodies of the edible mushroom *Hypsizygus marmoreus* when its pH was lowered. The hemolytic compound was purified using the modified Bligh and Dyer method followed by chromatography using reversed phase and silica gel columns. Structural analyses of the purified hemolytic compound were performed using NMR and ESI-MS. The deduced structure indicated a *trans*,*trans*-5,8-docosadienoic acid calcium salt. Although numerous proteinous hemolysins from various mushrooms have been described, the current study is the first to report on a low-molecular-weight hemolytic compound derived from an *H. marmoreus* extract.**

***Keywords:*** Docosadienoic acid, hemolysis, *Hypsizygus marmoreus*, mushroom

## 1. Introduction

Hemolytic proteins (hemolysins) have been identified in various mushroom fruiting bodies (*1*) such as aegerolysin in *Agrocybe aegerita* (*2*), *flammutoxin* in *Flammulina velutipes* (*3*), pleurotolysin and ostreolysin in *Pleurotus ostreatus* (*4,5*), erylysins in *P. eryngii* (*6*), nebrodeolysin in *P. nebrodensis* (*7*), schizolysin in *Schzophyllum commune* (*8*), and volvatoxin in *Volvarilla volvacea* (*9*). Although some mushroom hemolysins have been reported to recognize particular lipids comprising target cell membranes (*4,5,10,11*) and/or to form pores on those membranes (*10,12,13*), the physiological significance of hemolysins or cell membrane disruption in the life cycle of mushrooms has yet to be determined.

*Hypsizygus marmoreus* (buna-shimeji, or brown beech mushroom) is an edible mushroom. Proteinous hemolysins from other mushrooms have been identified, but a proteinous hemolysin from *H. marmoreus* has not been described thus far. A previous study by the current authors indicated that an extract of *H. marmoreus* fruiting bodies exhibited hemolytic activity against sheep red blood cells (RBCs) when the extract was incubated at a pH of 5.5 (*14*). The same study reported that the hemolytic compound exhibited lipid-like characteristics since the compound was water-insoluble and it was fractionated in a chloroform layer by the Bligh and Dyer method (*14*). The current study purified that hemolytic compound and it determined the structure of that compound.

## 2. Materials and Methods

### 2.1. *Fruiting bodies and reagents*

Fruiting bodies of *H. marmoreus* were purchased from a local market. Excised fruiting bodies were freeze-dried and stored at –30°C until use. Sheep blood was obtained from Nippon Bio-Supp Center, Tokyo, Japan. All chemicals used were of analytical grade.

### 2.2. *Hemolysis assay*

Hemolytic activity was determined as described previously (*14*). Briefly, sheep RBCs were washed with phosphate-buffered saline (PBS) three times. A suspension of sheep RBCs (0.1 mL) was mixed with 1.9 mL of distilled water in order to cause osmotic lysis, and absorbance was measured at 541 nm using a

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UV-Vis spectrophotometer (UVmini 1240; Shimadzu, Kyoto, Japan). PBS was added to the suspension so that the absorbance at 541 nm would be 0.500.

A reaction mixture (2 mL) containing 0.1 mL of suspended sheep RBCs prepared as described above, PBS, and a sample was incubated at 37°C for 10 min. A positive control was prepared by mixing 0.1 mL of suspended sheep RBCs with 1.9 mL of distilled water, and a negative control was prepared by similarly mixing the sheep RBCs with PBS. After centrifugation at 2,200 rpm for 5 min at 4°C, the absorbance of the supernatant was measured at 541 nm. One hundred percent lysis was defined as the absorbance of the supernatant obtained from osmotically lysed cells (the positive control) (*5*).

### 2.3. *Generation and preparation of a crude hemolytic compound*

Pieces of freeze-dried fruiting bodies of *H. marmoreus* (54 g) were ground and sonicated 10 times for 2 sec in 10 mM phosphate buffer (pH 7.3). The homogenate was centrifuged at 12,000 rpm for 30 min. In order to generate the hemolytic compound, the extract was mixed with an equal volume of a 0.1 M acetate buffer (pH 5.5) and incubated at 37°C for 30 min. The mixture was then centrifuged at 12,000 rpm for 20 min at 4°C. The precipitate was dissolved in ethanol and subjected to a modified Bligh and Dyer method with ethanol instead of methanol as described previously (*14,15*). The resulting fat-soluble fraction was dried in vacuo and dissolved in 50% ethanol.

### 2.4. *Purification of a hemolytic compound via column chromatography*

The sample dissolved in 50% ethanol was loaded onto a Strata C18-E cartridge column (500 mg/6 mL; Phenomenex, Torrance, CA, USA) equilibrated with the same solvent. The column was washed with 50% ethanol, and then stepwise elution was performed with 75 and 100% ethanol. Elution was monitored at 210 nm. Fraction size was 4 mL. Fractions with hemolytic activity were pooled and dried *in vacuo*.

The sample was subsequently dissolved in *n*-hexane/diethyl ether/acetic acid (80: 30: 1, v/v) and passed through a Wakogel C-200 column ($\phi$1.2 × 46 cm; Wako Pure Chemical Industries, Osaka, Japan) equilibrated with the same solvent system at a flow rate of 0.6 mL/min. The fractions (1 mL each) were collected, and the absorbance at 210 nm was measured. The hemolytic active fractions were pooled and subjected to rechromatography under the same conditions.

### 2.5. *Thin layer chromatography (TLC)*

TLC was performed with high-performance TLC silica gel 60 plates (Merck, Kenilworth, NJ, USA) and chloroform/methanol/water (60: 35: 6, v/v) or *n*-hexane/diethyl ether/acetic acid (80: 30: 1, v/v) as a development solvent. Lipids were detected with primulin staining, phosphomolybdic acid staining, and iodine vapor (*16-19*). TLC plates were also subjected to a hemolysis assay to detect hemolytic compounds as described previously (*20*).

### 2.6. *Structural analyses of the hemolytic compound*

Mass spectrometry analysis of the hemolytic compound was performed with a JMN-T100LP electrospray ionization mass spectrometer (JEOL, Tokyo, Japan). Electron probe microanalysis (EPMA) to detect carbon and calcium was performed on a silicon wafer using an EPMA-1610 electron probe microanalyzer (Shimadzu, Kyoto, Japan). Samples were dissolved in 0.1-0.2 mL of ethanol and subjected to the analyses.

Nuclear magnetic resonance (NMR) was analyzed using a 500 MHz Avance III HD NMR Spectrometer (Bruker, Bremen, Germany) at 300 K. Spectra were obtained from 6 mg of the purified hemolytic compound in 5-mm tubes (Kusanokagaku, Tokyo, Japan). The sample was dissolved in 0.45 mL of deuterated methanol (99.8%, Acros Organics, Geel, Belgium) containing tetramethylsilane ($\delta$H = 0.00) as an internal reference. Data from one-dimensional (proton, carbon, DEPT135; distortionless enhancement by polarization transfer) and two-dimensional homonuclear (correlation spectroscopy (COSY); nuclear Overhauser effect spectroscopy (NOESY)) and H-detected heteronuclear (heteronuclear multiple-quantum coherence (HMQC); heteronuclear multiple bond coherence (HMBC)) experiments were recorded and processed using the software TopSpin 3.5 pl 5 (Bruker).

## 3. Results and Discussion

A hemolytic fat-soluble fraction was prepared from an extract of *H. marmoreus* fruiting bodies by lowering its pH and subjecting the extract to a modified Bligh and Dyer method as described in the Materials and Methods. To purify the hemolytic compound from the fraction, C18 cartridge column chromatography was performed as described in the Materials and Methods. As shown in Figure 1A, substances detected at 210 nm were eluted with 50% ethanol, and strong hemolytic activity was evident with 75% ethanol. Weak activity was also noted in the eluent with 100% ethanol.

The major active fraction was then subjected to silica gel column chromatography as described in the Materials and Methods. As shown in Figure 1B, hemolytic activity was detected as a single peak. The active fraction was subjected to rechromatography for further purification. TLC analysis indicated that the final active fraction produced a single spot detected

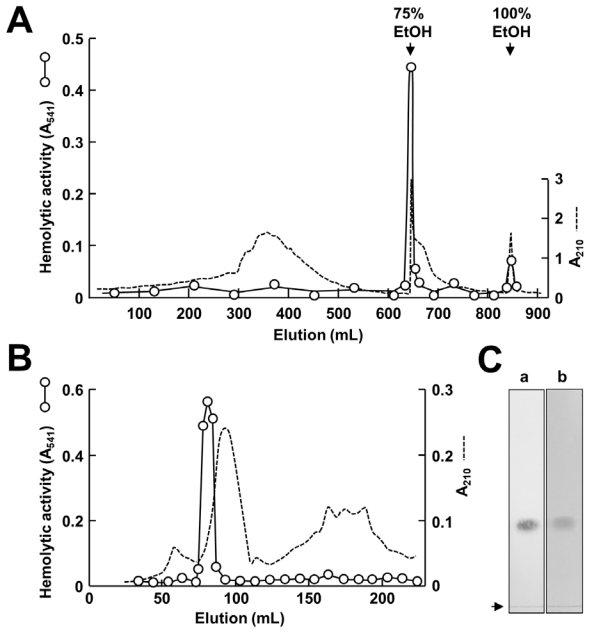



**Figure 1. Purification of a hemolytic compound *via* reversed phase and silica column chromatography. (A)** Chromatogram when using the Strata C18-E cartridge column. **(B)** Chromatogram when using the Wakogel C-200 column. Chromatography was performed as described in the Materials and Methods. **(C)** TLC analysis of a hemolytic compound purified *via* rechromatography with a silica gel column. *n*-hexane/diethyl ether/acetic acid (80: 30: 1, v/v) was used as a development solvent. Detection was performed using phosphomolybdic acid staining (lane a) and iodine vapor (lane b). An arrow indicates the origin.

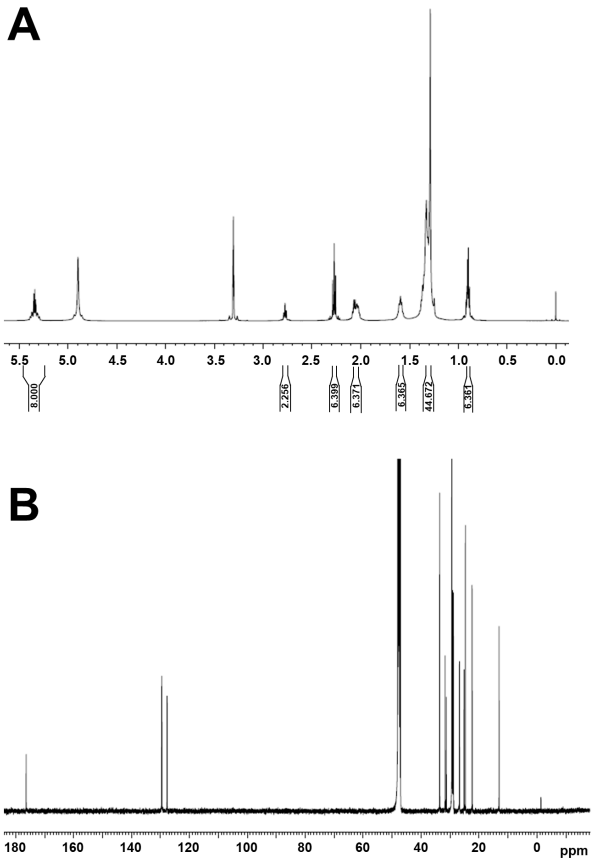



**Figure 2. NMR spectra. (A)** $^{1}$H NMR (500 MHz), **(B)** $^{13}$C NMR (125 MHz).

with phosphomolybdic acid staining and iodine vapor (Figure 1C). During this purification, 8.8 mg of the purified hemolytic compound was obtained from 1,360 mg of the fat-soluble substance (data not shown).

EPMA qualitatively detected calcium in the purified hemolytic sample by EPMA (data not shown). ESI-MS indicated an *m/z* of 710.5. The hemolytic compound was subjected to NMR analyses to determine its structure. $^{1}$H and $^{13}$C NMR data are summarized in Figure 2 and Table 1.

The relative configuration of the hemolytic compound was determined using NOESY NMR. Long-range NOE interactions between H-4 and H-6, H-8 and H-10, H-7 and H-9, H-5 and H-7 were observed (Figure 3). The deduced structure of the hemolytic compound indicated a calcium salt of *trans*,*trans*-5,8-docosadienoic acid (Figure 4).

The current study revealed that the hemolytic compound found in a low pH extract of the fruiting bodies of *H. marmoreus* was a *trans*,*trans*-5,8-docosadienoic acid. Proteinous hemolysins have been identified from various mushrooms, and these proteinous hemolysins are active in the first extract with buffers at a neutral pH (*1-9*). In contrast, the hemolytic compound from *H. marmoreus* was a type of fatty acid identified when the mushroom extract had a low pH (*14*). This may be the first low-molecular-weight compound found by means of assaying the hemolytic activity of mushroom components.

**Table 1. $^{1}$H NMR and $^{13}$C NMR data**

| Position | $^{1}$H NMR (integration of protons) | $^{13}$C NMR |
|---|---|---|
| 1 (COOH) | | 176.2, 176.3 |
| 2 | 1.59 (2H), 2.27 (2H) | 33.3, 33.5 |
| 3 | 1.59 (2H), 2.27 (2H) | 24.6, 24.6 |
| 4 | 1.34 (2H), 1.59 (2H) | 28.9, 28.9 |
| 5 | 5.34 (2H) | 129.4, 129.4 |
| 6 | 5.33 (2H) | 129.5, 129.5 |
| 7 | 2.27 (2H), 2.76 (2H) | 25.1, 25.1 |
| 8 | 5.35 (2H) | 127.6, 127.6 |
| 9 | 5.36 (2H) | 127.7, 127.7 |
| 10 | 2.03 (4H) | 26.7, 26.7 |
| 11 | 1.31 (2H), 2.06 (2H) | 29.1, 29.1 |
| 12 | 1.29 (4H) | 29.4, 29.4 |
| 13 | 1.29 (4H) | 29.3, 29.3 |
| 14-16 | 1.29-1.37 (12H) | 28.7, 28.8, 28.8, 28.9, 28.9, 29.2 |
| 17 | 1.34 (4H) | 29.0, 29.1 |
| 18 | 1.29 (4H) | 26.7, 26.7 |
| 19 | 1.29 (4H) | 29.4, 29.4 |
| 20 | 1.29 (4H) | 31.2, 31.6 |
| 21 | 1.32 (4H) | 22.2, 22.3 |
| 22 ($CH_3$) | 0.90 (6H) | 13.0, 13.1 |

Although fatty acids are known to potentially cause hemolysis by disrupting the cell membrane structure, the hemolytic fatty acid from *H. marmoreus* was

uniquely found in a fruiting body extract. In a previous study by the current authors, hemolytic activity was not detected when an extract was heated before its pH was lowered, but activity was evident when the extract was heat-stable after its pH was lowered (*14*). Therefore, some precursor substances may exist in fruiting bodies and some enzymes may release hemolytic fatty acids from those precursors. A previous study by the current authors indicated that hemolytic activity was $Ca^{2+}$-dependent and effectively inhibited in the presence of PMSF (which is a serine protease inhibitor) when the pH of an extract was lowered (*14*). Since the hemolytic compound examined in the current study was a fatty acid, some serine esterases/lipases possessing a catalytically active serine residue in the active site (*21*) might cleave the hemolytic fatty acid from a precursor.

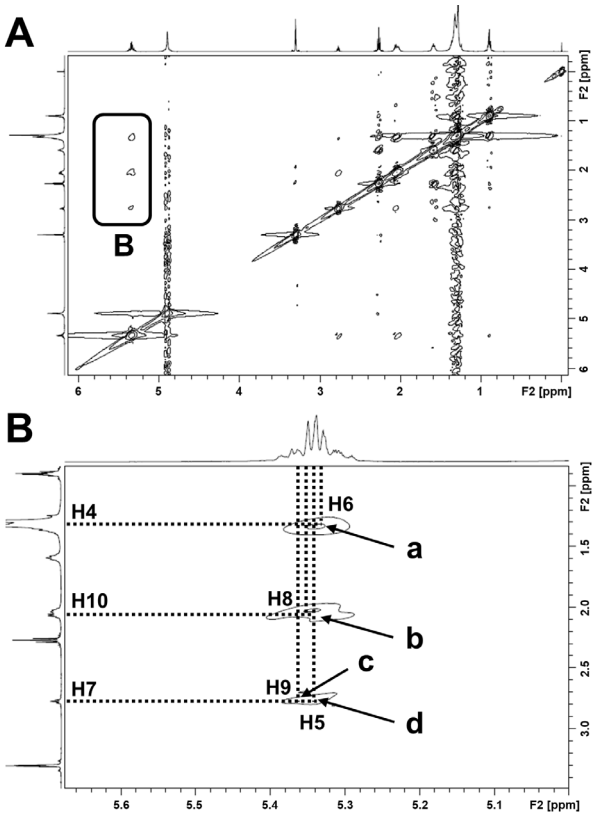
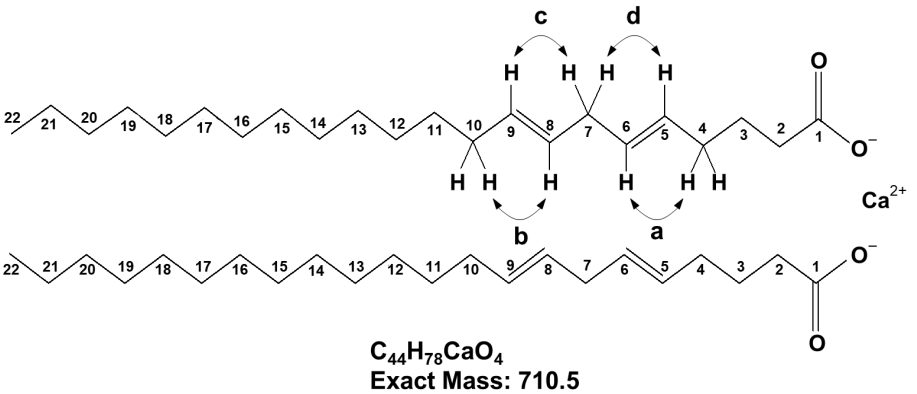



**Figure 3. NOESY Spectra.** Panel **A** shows the whole spectrum, and the indicated area is expanded in panel **B**. Arrows **a-d** indicate the long-range NOE interactions between H-4 and H-6, H-8 and H-10, H-7 and H-9, and H-5 and H-7, respectively.

Numerous types of fatty acids, including a small amount of docosadienoic acid ($C_{22:2}$), have been found in various mushrooms (*22-25*). Curiously, only *trans*,*trans*-5,8-docosadienoic acid was identified as a major hemolytic compound in the current study. Although other hemolytic substances were detected in the fat-soluble fraction according to TLC analysis and reversed-phase column chromatography, the docosadienoic acid was a major component purified as a hemolytic compound. One possibility is that the fruiting bodies of *H. marmoreus* might contain this type of fatty acid in abundance. Although 5,8-docosadienoic acid is a unique fatty acid in terms of the positions of double bonds, Δ5- and Δ8-fatty acid desaturases have been found in *H. marmoreus* (*26,27*). Another possibility is that enzymes acting to release hemolytic fatty acids might be specific to this type of fatty acid. Why the fatty acid calcium salt was obtained as a hemolytic compound in this study is still not known. Hydrogen ions in the fatty acid might be exchanged for calcium ions in the extract when its pH is lowered. Further study of the precursors and the enzymes such as esterases/lipases in fruiting bodies of *H. marmoreus* may be needed.

Mushroom hemolysins are thought to protect a fungus from outside factors or to participate in fusion of mycelia in the life cycle of basidiomycetes (*1*). The hemolytic compound found in the current study is not thought to exist as a free active substance. If its appearance is regulated in the life cycle of *H. marmoreus*, the current findings might represent a new step in determining the physiological role of hemolytic components in basidiomycetes.




**igure 4. Deduced structure of the hemolytic compound purified from a low pH-treated extract of *H. marmoreus* fruiting bodies.** NOE interaction; **a** (H-4 and H-6), **b** (H-8 and H-10), **c** (H-7 and H-9), **d** (H-5 and H-7).

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