Brief Report

DOI: 10.5582/bst.2018.01108

pH-Dependent exhibition of hemolytic activity by an extract of *Hypsizygus marmoreus* fruiting bodies

Kohsuke Saito[1], Syohto Hazama[1], Yoshiki Oda[2], Munehiro Nakata[1,*]

[1] *Department of Applied Biochemistry, Tokai University, Hiratsuka, Kanagawa, Japan;*
[2] *Technology Joint Management Office, Research Promotion Division, Tokai University, Hiratsuka, Kanagawa, Japan.*

Summary **The current study found that an extract from the fruiting bodies of the edible mushroom *Hypsizygus marmoreus* exhibited hemolytic activity against sheep red blood cells when its pH was lowered. Although hemolytic activity was not detected when an extract had a neutral pH, an extract with a low pH exhibited potent hemolytic activity. The maximal hemolytic activity was exhibited by an extract with a pH of 5.5. A heat-treated extract did not exhibit hemolytic activity before its pH was lowered, and that activity was inhibited in the presence of PMSF and EDTA. The turbidity of the extract increased during lowering of its pH, and the precipitate fraction exhibited hemolytic activity. Fractionation by a modified Bligh and Dyer method and TLC analyses suggested that a hemolytic compound in the extract might be a type of lipid. These results suggest that a hemolytic lipid-like compound in an extract of *H. marmoreus* fruiting bodies may be released by a non-active precursor substance(s) through metalloenzyme(s) while the extract has a low pH.**

Keywords: Hemolysis, hemolysin, *Hypsizygus marmoreus*, mushroom

1. Introduction

Higher Basidiomycetes mushrooms contain biologically active compounds in their fruiting bodies (*1,2*). Extracts prepared from the fruiting bodies of various mushrooms have been found to contain substances that act against red blood cells (RBCs) (*3,4*). These substances include hemolytic proteins (hemolysins), which cause the lysis of RBCs, and carbohydrate-binding proteins (lectins), which cause RBC agglutination. The carbohydrate-binding specificity of mushroom lectins has been ascertained, but few studies have examined mushroom hemolysins. Nonetheless, some mushroom hemolysins have been reported to assume an oligomeric structure, resulting in holes in the membrane of RBCs (*5*) similar to those created by the membrane attack complex of the complement system (*6,7*) or perforin produced by natural killer T cells (*8,9*). However, the physiological significance of hemolysins or cell membrane disruption in the life cycle of basidiomycetes has yet to be determined.

Hypsizygus marmoreus (buna-shimeji or brown beech mushroom) is an edible mushroom. Although a lectin from the mushroom (*H. marmoreus* lectin) has been identified (*10*), hemolysins from the mushroom and the mushroom's hemolytic activity have not been described. The current study examined the hemolytic activity of an extract of *H. marmoreus* fruiting bodies during lowering of its pH.

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. Phenylmethylsulfonyl fluoride (PMSF) and leupeptin were from MP Bio Japan, Tokyo, Japan, and ethylenediaminetetraacetic acid (EDTA) was from Dojindo Laboratories, Kumamoto, Japan. All chemicals used were of analytical grade.

Released online in J-STAGE as advance publication May 29, 2018.

**Address correspondence to:*
Dr. Munehiro Nakata, Department of Applied Biochemistry, Tokai University, Hiratsuka, Kanagawa 259-1292, Japan.
E-mail: nak@tsc.u-tokai.ac.jp

2.2. *Extract preparation and adjustment of its pH*

Pieces of freeze-dried fruiting bodies of *H. marmoreus* were grinded 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. The supernatant obtained was used as an extract.

The extract was mixed with an equal volume of a 0.1 M buffer at a pH range of 3.0-10.0 and incubated at 37°C for 30 min. The following buffers were used: a citrate buffer with a pH of 3.0 or 4.0, an acetate buffer with a pH of 5.0 or 5.5, a phosphate buffer with a pH of 6.0, 7.0, or 8.0, and a carbonate buffer with a pH of 9.0 or 10.0. The turbidity of the mixture was measured at 660 nm using a UV-Vis spectrophotometer (UVmini 1240; Shimadzu, Kyoto, Japan).

Inhibition of the hemolytic activity of an extract was examined while lowering its pH in the presence of 10 mM EDTA, PMSF, or 20 μg/mL of leupeptin. Effects of divalent metal ions were also examined using various metal chlorides at a concentration of 20 mM in the presence of 10 mM EDTA.

2.3. *Assay of hemolytic activity*

Sheep RBCs were used to determine hemolytic activity. The cells 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. 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) (*11*). One unit was defined as the amount of a hemolytic compound causing 50% hemolysis.

2.4. *Fractionation by a modified Bligh and Dyer method*

Pieces of freeze-dried fruiting bodies of *H. marmoreus* (54 g) were used to obtain the hemolytic compound. An extract prepared as described above was treated with 0.1 M acetate buffer (pH 5.5) and incubated at 37°C for 30 min. After centrifugation at 12,000 rpm for 20 min, the precipitate was dissolved in ethanol. A fat-soluble fraction was obtained with a modified solvent system using the Bligh and Dyer method (*12*) with ethanol instead of methanol. The sample dissolved in ethanol was mixed with one part chloroform and 0.9 parts distilled water. After vigorous vortexing, the mixture was centrifuged. The lower layer was mixed with the upper layer of a mixture of chloroform/ethanol/water (1:1:0.9, v/v) followed by vortexing and centrifugation again. The resulting lower layer was evaporated under reduced pressure and the resulting residue was dissolved in ethanol.

2.5. *Thin layer chromatography (TLC)*

TLC was performed with TLC plates (Merck, Kenilworth, NJ, USA) and chloroform/methanol/water (60:35:6, v/v) as a development solvent. Lpids were detected with primulin staining and iodine vapor (*13-16*). TLC plates were also subjected to a hemolysis assay to detect hemolytic compounds as described elsewhere (*17*).

2.6. *Data analysis*

Data were analyzed using Student's *t*-test with the software StatMate III (ATMS, Tokyo, Japan). A p value less than 0.05 was considered significant.

3. Results and Discussion

In preliminary experiments, hemolytic activity was detected in an acid-treated extract of *H. marmoreus* fruiting bodies but not in a neutral extract (Data not shown). Therefore, the relationship between the exhibition of hemolytic activity and the pH of the extract was examined. When the pH of the extract was adjusted at 37°C for 30 min, hemolytic activity was noted in acidic extracts but not in neutral or basic extracts (Figure 1). The maximal hemolytic activity was exhibited by an extract with a pH of 5.5.

Extracts with a low pH exhibited hemolytic activity and also had increased turbidity (Figure 1). A turbid extract with a pH of 5.5 was centrifuged at 12,000 rpm for 20 min in order to separate the supernatant and precipitate fractions. Potent hemolytic activity was exhibited by the precipitate fraction that was dissolved in ethanol (data not shown). In contrast, hemolytic activity was not detected in the supernatant fraction while potent hemagglutinating activity was noted (data not shown). This may have been caused by *H. marmoreus* lectin (*10*). This finding suggests that a water-insoluble hemolytic compound is present in the extract during its incubation at a low pH.

The effect of heat on the exhibition of hemolytic activity was examined. A boiled extract did not exhibit hemolytic activity or an increase in turbidity when its pH was 5.5 (Figure 2A). In contrast, the precipitate fraction that was obtained from an extract with a pH of 5.5 retained sufficient hemolytic activity even if the fraction was heat-treated (Figure 2B). These results

suggest that the hemolytic compound is heat-stable and that heat-labile metabolism may account for the hemolytic activity exhibited by the compound.

The effect that inhibitors had on the generation of the hemolytic compound was examined. Extracts were prepared with a neutral pH in the presence of inhibitors and then their pH was lowered to 5.5. As shown in Figure 3, hemolytic activity during lowering of the pH was prevented by PMSF and the divalent cation chelator EDTA but not by leupeptin. Hemolytic activity inhibited by EDTA markedly returned in the presence of Ca^{2+}, though other cations failed to sufficiently restore that activity (Figure 3B).

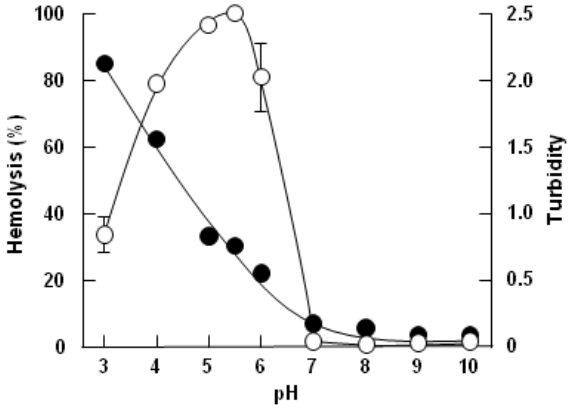

Figure 1. Effect of pH on the exhibition of hemolytic activity by and the turbidity of an extract of *H. marmoreus* fruiting bodies. An extract was incubated at 37°C for 30 min at the pH indicated. The hemolytic activity of aliquots was measured (open circle) and turbidity was determined (closed circle) as described in the Materials and Methods.

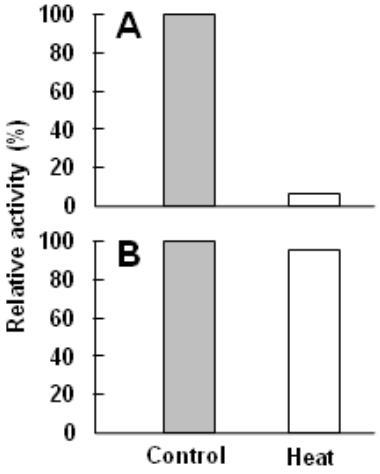

Figure 2. Effect of heat-treatment on the hemolytic activity of extracts before or after lowering pH. An extract prepared at a pH of 7.3 was boiled for 10 min and then its pH was lowered to 5.5 **(A)** or its pH was lowered to 5.5 and then it was boiled **(B)**. The samples were subjected to a hemolytic assay. Controls were produced by omitting boiling.

To characterize the nature of the hemolytic compound, the precipitate fraction obtained from an extract with a pH of 5.5 was further fractionated using a modified Bligh-Dyer method as described in the Materials and Methods. As shown in Figure 4A, potent hemolytic activity was detected in the lower

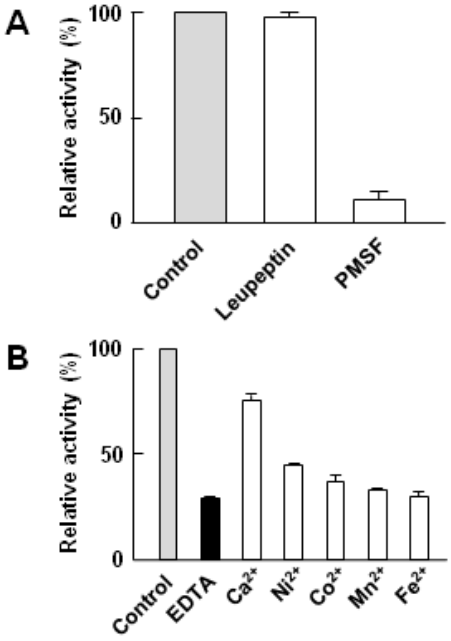

Figure 3. Effect of inhibitors of hemolytic activity on extracts with a low pH. (A) Extracts prepared at a pH of 7.3 were separately pretreated with or without 10 mM PMSF and 20 μg/mL of leupeptin. **(B)** Extracts were pretreated with or without 10 mM EDTA followed by incubation with 20 mM of the metal ions indicated. The pretreated extracts were incubated at a pH of 5.5 and then subjected to a hemolytic assay.

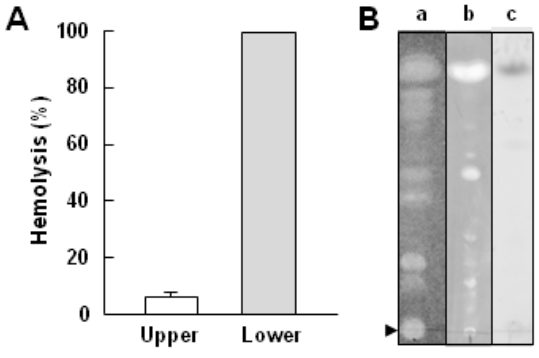

Figure 4. Fractionation of hemolytic activity using a modified Bligh and Dyer method and TLC. (A) A precipitate fraction was fractionated by using a modified Bligh and Dyer method as described in the Materials and Methods. Upper and lower layer fractions dissolved in water and ethanol, respectively, were subjected to a hemolysis assay. **(B)** The lower layer fraction was developed on TLC plates using a solvent system of chloroform/methanol/water (60:35:6, v/v). Detection was performed by primulin staining (lane a), a hemolysis assay (lane b), and iodine vapor (lane c). An arrowhead indicates the origin.

layer (lipid fraction) but not in the upper layer (aqueous fraction), suggesting that the hemolytic component is a type of lipid. Primulin staining after TLC development revealed that the lower layer fraction contained various components (Figure 4B, lane a). A hemolysis assay of TLC plates revealed hemolytic activity at several places on the plate (Figure 4B, lane b). Potent activity was noted at the same site where a spot was produced by iodine vapor (Figure 4B, lane c). These results suggest that the hemolytic compound in an extract of *H. marmoreus* fruiting bodies with a low pH may be a lipid-like substance.

Proteinous hemolysins have been found in various mushrooms, including aegerolysin in *Agrocybe aegerita* (*18*), flammutoxin in *Flammulina velutipes* (*19*), pleurotolysin and ostreolysin in *Pleurotus ostreatus* (*11,20*), erylysins in *P. eryngii* (*21*), nebrodeolysin in *P. nebrodensis* (*22*), schizolysin in *Schzophyllum commune* (*23*), and volvatoxin in *Volvarilla volvacea* (*24*). These hemolysins were active when the first extract was adjusted to a neutral pH with buffers. However, a proteinous hemolysin from *H. marmoreus* has yet to be described. Lowering of the pH of an extract of *H. marmoreus* was essential for it to exhibit hemolytic activity (Figure 1), which presumably explains why this activity was not been noted thus far.

That said, the hemolytic compound found in the current study may be a lipid-like substance but not a proteinous hemolysin as has been found in various mushrooms. Some lipids such as fatty acids are known to non-specifically damage cells by disrupting their membrane structure. In fact, the compound found in the current study non-specifically caused hemolysis of rabbit and horse RBCs as well as sheep RBCs (data not shown).

Hemolytic activity was not detected in an extract heated before its pH was lowered, but activity was detected in a heat-stable extract after its pH was lowered (Figure 2). Therefore, a non-active precursor substance(s) may be present in fruiting bodies and a heat-labile enzyme(s) may cause that precursor(s) to release a heat-stable hemolytic compound. Since hemolytic activity was inhibited in the presence of EDTA and that activity resumed with the addition of Ca^{2+} (Figure 3B), enzyme action is presumably Ca^{2+}-dependent. In addition, PMSF (a known serine protease inhibitor) was an effective inhibitor of hemolytic activity during lowering of the extract's pH (Figure 3A). However, leupeptin (a competitive inhibitor of serine protease) did not inhibit hemolytic activity (Figure 3A). Therefore, a metalloenzyme possessing a catalytically active serine residue in its active site, but not serine protease, might be responsible for the formation of a hemolytic compound. The structure of the hemolytic lipid-like compound(s) and the precursor(s) and the enzymes in *H. marmoreus* fruiting bodies need to be studied further.

The role of mushroom hemolysins is somewhat unclear. Since hemolysins influence the cell membrane structure and lyse cells, hemolysins may provide protection from outside factors or participate in the fusion of mycelia in the life cycle of basidiomycetes (*3*). The hemolytic lipid-like compound found in the current study is not thought to exist as a free active substance in the fruiting bodies of *H. marmoreus*. If hemolytic activity is regulated in the life cycle of *H. marmoreus*, the current study might provide new evidence of the physiological role of hemolytic components in basidiomycetes.

In conclusion, a non-active precursor substance(s) may release a hemolytic lipid-like compound from an extract of *H. marmoreus* fruiting bodies through metalloenzyme(s) while the extract has a low pH.

References

1. Wasser SP, Weis AL. Medicinal properties of substances occurring in higher basidiomycetes mushrooms: Current perspectives. Int J Med Mushrooms. 1999; 1:31.
2. Wasser SP. Medicinal mushroom science: Current perspectives, advances, evidences, and challenges. Biomed J. 2014; 37:345-356.
3. Nayak AP, Green BJ, Beezhold DH. Fungal hemolysins. Med Mycol. 2013; 51:1-16.
4. Hassan MA, Rouf R, Tiralongo E, May TW, Tiralongo J. Mushroom lectins: Specificity, structure and bioactivity relevant to human disease. Int J Mol Sci. 2015; 16:7802-7838.
5. Sepčić K, Berne S, Rebolj K, Batista U, Plemenitas A, Sentjurc M, Macek P. Ostreolysin, a pore-forming protein from the oyster mushroom, interacts specifically with membrane cholesterol-rich lipid domains. FEBS Lett. 2004; 575:81-85.
6. McCormack R, de Armas L, Shiratsuchi M, Podack ER. Killing machines: Three pore-forming proteins of the immune system. Immunol Res. 2013; 57:268-278.
7. Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement system Part I - Molecular mechanisms of activation and regulation. Front Immunol. 2015; 6:262.
8. Voskoboinik I, Whisstock JC, Trapani JA. Perforin and granzymes: Function, dysfunction and human pathology. Nat Rev Immunol. 2015; 15:388-400.
9. Gilbert RJ. Protein-lipid interactions and non-lamellar lipidic structures in membrane pore formation and membrane fusion. Biochim Biophys Acta. 2016;1858:487-499.
10. Suzuki T, Abe T, Umehara K, Choi J-H, Hirai H, Dohra H, Kawagishi H. Purification and characterization of a lectin from the mushroom *Hypsizigus marmoreus*. Mycoscience. 2015; 56:359-363.
11. Tomita T, Noguchi K, Mimuro H, Ukaji F, Ito K, Sugawara-Tomita N, Hashimoto Y. Pleurotolysin, a novel sphingomyelin-specific two-component cytolysin from the edible mushroom *Pleurotus ostreatus*, assembles into a transmembrane pore complex. J Biol Chem. 2004; 279:26975-26982.
12. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol. 1959; 37:911-917.

13. Vioque E, Holman RT. Characterization of the ketodienes formed in the oxidation of linoleate by lipoxidase. Arch Biochem Biophys. 1962; 99:522-528.
14. Feizi T, Stoll MS, Yuen CT, Chai W, Lawson AM. Neoglycolipids: probes of oligosaccharide structure, antigenicity, and function. Methods Enzymol. 1994; 230:484-519.
15. Zarzycki PK, Bartoszuk MA, Radziwon AI. Optimization of TLC detection by phosphomolybdic acid staining for robust quantification of cholesterol and bile acids. J Planar Chromatogr. 2006; 19:52-57.
16. Fuchs B, Süss R, Teuber K, Eibisch M, Schiller J. Lipid analysis by thin-layer chromatography--a review of the current state. J Chromatogr A. 2011; 1218:2754-2774.
17. Sharma OP, Kumar N, Singh B, Bhat TK. An improved method for thin layer chromatographic analysis of saponins. Food Chem. 2012; 132:671-674.
18. Berne S, Krizaj I, Pohleven F, Turk T, Macek P, Sepcić K. Pleurotus and Agrocybe hemolysins, new proteins hypothetically involved in fungal fruiting. Biochim Biophys Acta. 2002; 1570:153-159.
19. Bernheimer AW, Oppenheim JD. Some properties of flammutoxin from the edible mushroom *Flammulina velutipes*. Toxicon. 1987; 25:1145-1152.
20. Sepcić K, Berne S, Potrich C, Turk T, Macek P, Menestrina G. Interaction of ostreolysin, a cytolytic protein from the edible mushroom *Pleurotus ostreatus*, with lipid membranes and modulation by lysophospholipids. Eur J Biochem. 2003; 270:1199-1210.
21. Shibata T, Kudou M, Hoshi Y, Kudo A, Nanashima N, Miyairi K. Isolation and characterization of a novel two-component hemolysin, erylysin A and B, from an edible mushroom, *Pleurotus eryngii*. Toxicon. 2010; 56:1436-1442.
22. Lv H, Kong Y, Yao Q, Zhang B, Leng FW, Bian HJ, Balzarini J, Van Damme E, Bao JK. Nebrodeolysin, a novel hemolytic protein from mushroom Pleurotus nebrodensis with apoptosis-inducing and anti-HIV-1 effects. Phytomedicine. 2009; 16:198-205.
23. Han CH, Zhang GQ, Wang HX, Ng TB. Schizolysin, a hemolysin from the split gill mushroom *Schizophyllum commune*. FEMS Microbiol Lett. 2010; 309:115-121.
24. Weng YP, Lin YP, Hsu CI, Lin JY. Functional domains of a pore-forming cardiotoxic protein, volvatoxin A2. J Biol Chem. 2004; 279:6805-6814.

(Received May 8, 2018; Revised May 19, 2018; Accepted May 24, 2018)