Pleurotolysin, a Novel Sphingomyelin-specific Two-component Cytolysin from the Edible Mushroom *Pleurotus ostreatus*, Assembles into a Transmembrane Pore Complex*

Received for publication, March 9, 2004, and in revised form, April 13, 2004
Published, JBC Papers in Press, April 14, 2004, DOI 10.1074/jbc.M402676200

Toshio Tomita‡§, Kayoko Noguchi,‡ Hitomi Mimuro¶, Fumio Ukaji†, Kiyoshi Ito¶, Noriko Sugawara-Tomita‡, and Yohichi Hashimoto‡

From the ‡Department of Microbial Biotechnology, Graduate School of Agricultural Science, Tohoku University, Aoba-ku, Sendai 981-8555, Japan and ¶Department of Biochemistry and Molecular Biology, Faculty of Science, Saitama University, 255 Shimo-Ohkubo, Sakura-ku, Saitama 388-8570, Japan

Self-assembling, pore-forming cytolysins are illustrative molecules for the study of the assembly and membrane insertion of transmembrane pores. Here we purified pleurotolysin, a novel sphingomyelin-specific two-component cytolysin from the basidiocarps of *Pleurotus ostreatus* and studied the pore-forming properties of the cytolysin. Pleurotolysin consisted of non-associated A (17 kDa) and B (59 kDa) components, which cooperatively caused leakage of potassium ions from human erythrocytes and swelling of the cells at nanomolar concentrations, leading to colloid-osmotic hemolysis. Hemolytic assays in the presence of poly(ethylene glycol) with different hydrodynamic diameters suggested that pleurotolysin formed membrane pores with a functional diameter of 3.8–5.5 nm. Pleurotolysin-induced lysis of human erythrocytes was specifically inhibited by the addition of sphingomyelin-cholesterol liposomes to the extracellular space. Pleurotolysin A specifically bound to sphingomyelin-cholesterol liposomes and caused leakage of the internal carboxyfluorescein in concert with pleurotolysin B. Experiments including solubilization of pleurotolysin-treated erythrocytes with 2% (w/v) SDS at 25 °C and SDS-polyacrylamide gel electrophoresis/Western immunoblotting showed that pleurotolysin A and B bound to human erythrocytes in this sequence and assembled into an SDS-stable, 700-kDa complex. Ring-shaped structures with outer and inner diameters of 14 and 7 nm, respectively, were isolated from the solubilized erythrocyte membranes by a sucrose gradient centrifugation. Pleurotolysin A and B formed an SDS-stable, ring-shaped complex of the same dimensions on sphingomyelin-cholesterol liposomes as well.

Pore-forming cytolytic proteins are distributed in a wide variety of eukaryotic and prokaryotic organisms (1, 2). The self-assembling, pore-forming cytolysins are also illustrative molecules for the study of the assembly, membrane insertion, and molecular architecture of transmembrane pores (2–4). Several cytolytic proteins have been isolated from the basidiocarps of both toxic and edible mushrooms, and their pore-forming properties as well as cardiotoxicity and cytotoxicity were studied (5–9). Although physiological function of the mushroom cytolysins remains enigmatic, recent studies implied the involvement of hemolytic proteins in the fruiting initiation of some mushrooms. The *Aa-Pri1* gene encoding a 16-kDa putative protein has been shown to be specifically expressed in the fruiting initiation of the edible mushroom *Agrocybe aegerita* (10, 11). Aegerolysin, a 17-kDa hemolytic protein isolated from the basidiocarps of *A. aegerita*, has an identical N-terminal amino acid sequence with the predicted *Aa-Pri1* protein, and it was detected only in the primordia and immature fruiting bodies of the mushroom (11).

Previously, Bernheimer and Avigad (12) isolated a 12-kDa cytolysin from the basidiocarps of *Pleurotus ostreatus* and designated it pleurotolysin. They show that pleurotolysin has unique characteristics such as sphingomyelin-specific hemolytic activity and an unusual amino acid composition with the lack of seven amino acids commonly found in proteins. Although we detected hemolytic activity in the homogenate of the mushroom, we failed to isolate the 12-kDa hemolysin. Instead, we found that the hemolytic activity of the mushroom homogenate was lost by a gel filtration using Sephadex G-75, but it was recovered to the original level when all of the fractions were recombined. In addition, hemolytic activity of the mushroom homogenate decreased to 10% of the original level at 4 °C within a day unless protease inhibitors were added. The results suggested that *P. ostreatus* hemolysin consists of multiple components, and the pleurotolysin of Bernheimer and Avigad (12) could be a proteolytic product. Recently, ostreolysin was isolated as a 16-kDa hemolysin from the basidiocarps of *P. ostreatus*, which was suggested to be specifically expressed in primordia and fruiting bodies of the mushroom (11). In contrast to the sphingomyelin-specific pleurotolysin described by Bernheimer and Avigad (12), ostreolysin is inhibited by a series of lysophospholipids but not inhibited by sphingomyelin (11, 13).

In this study, we purified a novel sphingomyelin-specific cytolysin consisting of A (17 kDa) and B (59 kDa) components from the basidiocarps of *P. ostreatus* and designated it pleurotolysin (Ply) because of its sphingomyelin specificity. We stud-
Sphingomyelin-specific Two-component Cytolysin from Mushroom

Purification of Ply—All purification steps were carried out at 0–4 °C, except for high performance liquid chromatography, and Ply fractions were treated with protease inhibitors at each step. Human erythrocytes were used for hemolytic assay. Basidiocarps of *P. ostreatus* (1 kg, wet weight) were homogenized in 1 liter of 50 mM Tris-HCl buffer (pH 8.3) with 2% poly(ethyleneimine) hydrochloride, filtered using a 100-μm filter, and dialyzed against 10 mM Tris-HCl buffer (pH 8.0) for 30 min. After centrifugation at 600 × *g* for 30 min, the erythrocytes were collected by centrifugation at 22,000 × *g* for 10 min at 4 °C and washed twice with 5 mM Tris-HCl buffer (pH 7.2). The erythrocyte membranes were solubilized with 2% (v/v) SDS at 25 °C and were subjected to SDS-PAGE using a linear gradient gel of 3–22% (w/v) acrylamide followed by Western immunoblot using antiserum against Ply A and B. Sphingomyelin-cholesterol liposomes (0.2 μM of each) at 25 °C for 30 min. The liposomes were collected and washed twice with TBS. The washed liposomes were solubilized with 2% (w/v) SDS at 37 °C for 10 min and were subjected to SDS-PAGE and Western immunoblot.

**Isolation of the High Molecular Weight Complex**—Human erythrocytes (6 × 10^7 cells) were incubated with Ply A and B (500 μg of each) in 100 ml of TBS at 25 °C for 30 min. Erythrocyte membranes were collected and washed twice with 5 mM Tris-HCl buffer (pH 7.2). The washed cell membranes were treated by centrifugation using a Beckman SW40Ti rotor at 36,000 rpm at 4 °C for 1 h. The packed cell membranes were treated with 2% SDS (w/v) at 25 °C and loaded onto a 10–40% (w/v) sucrose gradient in 10 mM Tris-HCl buffer (pH 7.2) containing 0.1% SDS. Centrifugation was performed using a Beckman SW40Ti rotor at 32,000 rpm for 19 h at 4 °C. Thyroglobulin (669 kDa), apoferritin (480 kDa), and catalase (232 kDa) were used as molecular size markers.

**Electronic Microscopy**—Fractions containing the high molecular weight complexes were dialyzed against 10 mM Tris-HCl buffer (pH 7.2) containing 0.1% (w/v) SDS at 4 °C. The dialyzed samples were placed onto carbon-coated grids, washed briefly with 5 mM sodium phosphate buffer (pH 7.2), and stained negatively with 1% (w/v) sodium phosphotungstic acid (pH 7.2). Specimens were examined under a Hitachi electron microscope H-8100 (Tokyo, Japan) at an acceleration voltage of 80 kV.

**Miscellaneous**—Protein concentration was assayed as described by Bradford (18) using bovine serum albumin as a standard. The N-terminal amino acid sequence was analyzed using an ABI model 491 protein sequencer (PE/Applied Biosystems, Foster City, CA) (19). Osmotic pressure of the polyethylene glycol solution was measured at 25 °C using a micro-osmometer, model 3MO (Advanced Instruments, Needham Heights, MA). Phosphorus was assayed as described (20). Sphingomyelinase activity was assayed as described (21).

**Materials and Chemicals**—Basidiocarps of *P. ostreatus* were purchased from a farm and stored at −40 °C before use. Polyethylene glycols were from Wako Pure Chemicals (Osaka, Japan). Egg yolk phosphatidylcholine was from Nippon Oil and Fats Co., (Tokyo, Japan). For lipid electrophoresis, brain phosphatidylethanolamine and used as a standard. Phospholipid mixtures contained α,β-dimyristoyl phosphatidylglycerol, soybean phosphatidylinositol, and bovine cardiac muscle phosphatidylcholine were from Avanti Polar Lipids (Alabaster, AL). Cholesterol (Sigma) was crystallized twice in methanol. CF was purchased from Molecular Probes (Eugene, OR) and purified as described (22).

**RESULTS**

Purification of a Novel Two-component Hemolysin from the Basidiocarps of *P. ostreatus*—The hemolytically active extract from the basidiocarps of *P. ostreatus* was fractionated by column chromatography using DEAE-Cellulofine A-500, hydroxyapatite, and a phenyl Toyopearl 650M column. The column chromatography using the phenyl Toyopearl 650M column separated the hemolytically active hydroxyapatite fraction into the unadsorbed Ply A and the adsorbed Ply B fractions (results not shown). Approximately 130 and 20 mg proteins were obtained in the A and B fractions, respectively, from the basidiocarps (1 kg, wet weight). The Ply A fraction was further purified by passing it through a Toyopearl HW50S column, and the A fraction thus obtained gave a single protein band corresponding to 17 kDa on SDS-PAGE (Fig. 1A). In contrast, the B fraction obtained from the phenyl Toyopearl 650M column chromatography gave six major protein bands corresponding to 64, 59, 43, 41, 23, and 18 kDa on SDS-PAGE (Fig. 1A). On the basis of the following results, we assumed that the 41- and 18-kDa proteins derived from the 59-kDa protein through a limited proteolysis. The results of the activity assay demonstrated that the high molecular weight complex was separated into the A and B fractions by SDS-PAGE (Fig. 1A).

**Binding of Ply to Liposomes and Lipoprotein Lysates**—Multilamellar liposomes were prepared from phospholipid and cholesterol as described (17). The liposomes (0.1 μmol of lipids) were incubated with Ply A or B (10 μg of each) in 1 ml of Tris-buffered saline (TBS) at 25 °C for 30 min. The liposomes were washed twice with TBS and were subjected to SDS-PAGE after treatment with 2% SDS at 100 °C for 5 min. Protein bands were stained with Coomassie Brilliant Blue R-250 (CBB). To assay liposome lysis, carboxyfluorescein (CF)-loaded liposomes (0.5 nmol of lipids in 50 μl) were incubated with Ply A and B at 25 °C for 30 min in a microplate (Nunc, Roskilde, Denmark). Fluorescence of the released CF was measured using a microplate reader MTP32 (Corona Electric, Katsuda, Japan). 2% CF release was defined as the fluorescence intensity obtained upon exposure to 0.5% Triton X-100.

Assembly of Ply A and B into a High Molecular Weight Complex on Erythrocytes and Sphingomyelin-Cholesterol Liposomes—Erythrocytes (6 × 10^7 cells) were incubated with Ply A and B (5 and 0.5 μg of each for direct stain with CFBB or for immunostain, respectively) in 1 ml of TBS at 25 °C for 30 min. The erythrocytes were collected by centrifugation at 22,000 × *g* for 10 min at 4 °C and washed twice with 5 mM Tris-HCl buffer (pH 7.2). The erythrocyte membranes were solubilized with 2% (w/v) SDS at 25 °C and were subjected to SDS-PAGE using a linear gradient gel of 3–22% (w/v) acrylamide followed by Western immunoblot using antiserum against Ply A and B. Sphingomyelin-cholesterol liposomes (0.2 μM of each) at 25 °C for 30 min. The liposomes were collected and washed twice with TBS. The washed liposomes were solubilized with 2% (w/v) SDS at 37 °C for 10 min and were subjected to SDS-PAGE and Western immunoblot.

**Effect of Cytolysin Structure on Erythrocyte Lysis**—To examine whether the two-component cytolytic activity of Ply A and B is required for the lysis of erythrocytes by Ply A and B, we replaced either Ply A or B with a mixture of Ply A and B (1.6 μg of each) at 25 °C for 30 min. The erythrocytes were washed twice with TBS and subjected to SDS-PAGE. The lane containing the untreated erythrocytes was loaded with 2% SDS at 25 °C, and the lane containing the erythrocytes treated with 2% SDS at 100 °C for 5 min was loaded with 2% SDS at 37 °C. Protein bands were stained with Coomassie Brilliant Blue R-250 (CBB). To assay liposome lysis, carboxyfluorescein (CF)-loaded liposomes (0.5 nmol of lipids in 50 μl) were incubated with Ply A and B at 25 °C for 30 min in a microplate (Nunc, Roskilde, Denmark). Fluorescence of the released CF was measured using a microplate reader MTP32 (Corona Electric, Katsuda, Japan). 2% CF release was defined as the fluorescence intensity obtained upon exposure to 0.5% Triton X-100.
formance liquid chromatography using a DEAE-5PW column fractionated the B fraction into two Ply B peaks (Fig. 1B). The first Ply B peak gave a major protein band corresponding to 59 kDa, which was eluted in tandem with Ply B activity (Fig. 1B). In contrast, the second Ply B peak contained two major proteins of 41 and 18 kDa (Fig. 1B). (ii) Unless protease inhibitors were used at each purification step, the relative amount of the 59-kDa protein decreased in the B fraction, and relative amounts of the 41- and 18-kDa proteins increased (results not shown). To test this possibility, we raised specific antisera to the 59- and the 41-kDa proteins for Western immunoblot. Antiserum against the 59-kDa protein recognized all the protein bands corresponding to 59, 41, and 18 kDa, whereas antiserum against the 41-kDa protein reacted with the 59-kDa protein but not with the 18-kDa protein (Fig. 1C). Furthermore, N-terminal amino acid sequences of the 59-, 41-, and 18-kDa proteins (i.e. Ser-Gln-Ala-Gly-Asp-Arg-Thr-Leu-Asn-Asp-Val-Ile-Gln-, Phe-Asp-Asp-Val-Ile-Lys-Leu-Ser-Pro-Gln-Phe-Thr-His-Gly-Val-Gln-Ala-Ala-Leu-Ala-Lys-, and Ala-Gly-Asp-Arg-Leu-Aasn-Asp-Val-Ile-Gln-, respectively) indicated that the 59- and the 18-kDa proteins have identical N-terminal amino sequences, although the 18-kDa polypeptide has lost the first two amino acid residues. The results suggested that the 59-kDa protein is Ply B, and the 18- and 41-kDa proteins derived from the N- and C-terminal regions of the 59-kDa protein, respectively.

To identify Ply B, we attempted to purify the 59-, 41-, and 18-kDa proteins. The Ply B fraction obtained from the phenyl Toyopearl 650M column chromatography was electrofocused into two fractions corresponding to pH 6.8 and 7.0 (Fig. 2A; fractions I and II, respectively). Fraction I contained a single major protein of 59 kDa and minor ones of 41 and 18 kDa, whereas fraction II contained the major proteins of 41 and 18 kDa and a minor one of 59 kDa (results not shown). Fraction I was adsorbed on to a DEAE-5PW column, and two Ply B peaks were eluted with a linear gradient of NaCl (Fig. 2B). The 59-kDa protein, which was eluted as the major protein in the first peak, was purified to homogeneity by the second DEAE-5PW column chromatography (Fig. 2, D and F, lane I). The 41- and 18-kDa proteins were purified from fraction II using two steps of DEAE-5PW column chromatography (Fig. 2, C, E, and F, lane II). The purified 59-kDa protein and the mixture of the 41- and the 18-kDa proteins caused 50% lysis of human erythrocytes at 17 and 58 ng/ml, respectively, in concert with Ply A (1 μg/ml). Both the 59-kDa protein and the mixture of the 41- and 18-kDa proteins gave single peaks of absorbance at 280 nm corresponding to 60 kDa on the gel filtration using a TSKgel G-3000SW column (results not shown). Therefore, the 59-kDa protein existed as a monomer in solution, and the polypeptides of 41- and 18-kDa were non-covalently associated with each
other. Taken together with the results of the Western immunoblot (Fig. 1C) and the protein sequencing described above, the 59-kDa protein is Ply B, and Ply is the single hemolysin in the basidiocarps of P. ostreatus.

To assess the optimal molar ratio of Ply A to B for induction of hemolysis, the mixtures of Ply A and B in various molar ratios were assayed for hemolytic activity toward human erythrocytes. Ply exhibited the maximal hemolytic activity when the A and B components were mixed in a molar ratio of 3 (Fig. 3A). On the basis of the results, Ply A and B of the same protein concentration were mixed and used in the following experiments (where molar ratio of Ply A to B was 3.5) unless otherwise stated. Ply A and B caused 50% hemolysis when each of the components was present at 50 ng/ml (i.e. 2.9 and 0.85 nM, respectively), whereas neither Ply A nor B induced significant hemolysis at higher concentrations of up to 80 μg/ml without their counterpart (Fig. 3B).

The N-terminal 32 and 12 amino acid residues of Ply A and B were determined to be Ala-Tyr-Ala-Gln-Trp-Val-Ile-Ile-Ile-His-Azn-Val-Gly-Ser-Lys-Ser-Val-His-Lys-His-Lys-Leu-Leu-Lys-Pro-Ser-Trp-Gly-Lys-Leu-His-Ala- and Ser-Gln-Ala-Gly-Arg-Leu-Asn-Asp-Val-Ile-Gln, respectively. A similarity search on the DDBJ/GenBankTM/EBI nucleotide sequence data bases indicated that Ply B has no similar sequence with any other protein. In contrast, the N-terminal amino acid sequence of Ply A is identical with that of the P. ostreatus PriA protein (GenBankTM accession number AF331453-1), which is a predicted product from the nucleotide sequence of a mRNA from the mushroom. However, no information other than the nucleotide sequence is available for the P. ostreatus PriA protein. The N-terminal amino acid sequence of Ply A also exhibits 93 and 75% identity with those of P. ostreatus ostreolysin (GenBankTM accession number P83467) and A. aegerita Aa-Pri1 (GenBankTM accession number AF004297-1), respectively.

Cooperative Pore-forming Activity of Ply—When human erythrocytes were exposed to Ply A and B at 25 °C, intact disc shaped erythrocytes became swollen round shaped cells with a clear edge within 2–3 min, and thereafter the swollen cells released hemoglobins (Fig. 4A-C). Because swelling of cells is generally caused by an increased permeability of cell membranes, we assayed Ply-induced leakage of potassium ions and hemoglobins from human erythrocytes. >90% of the intracellular potassium ions leaked within 3 min (Fig. 4D). The Ptreated erythrocytes were swollen but not lysed in the presence of poly(ethylene glycol) 6000 (results not shown). The results suggested that Ply A and B cooperatively formed membrane pores to cause the colloid-osmotic burst of human erythrocytes, and the functional diameter of the pore was smaller than the hydrated diameter of hemoglobin but larger than that of a potassium ion. To estimate the functional diameter of the pores, we assayed the Ply-induced lysis of human erythrocytes in the presence of poly(ethylene glycol)s with different hydrodynamic diameters. Ply-induced hemolysis was not significantly affected by the addition of poly(ethylene glycol)s 200–1000 (Fig. 5). In contrast, Ply-induced hemolysis was partially inhibited by poly(ethylene glycol)s 2000, 3000, and 4000, and it was entirely suppressed by poly(ethylene glycol)s 6000, 8000, and 10,000 (Fig. 5). The hydrodynamic diameters of poly(ethylene glycol)s 4000 and 6000 were estimated to be 3.8 and 5.0 nm, respectively (15, 16). Similar experiments using neutral sugars showed that Ply lysed human erythrocytes in the presence of dextran 4, but it caused no hemolysis in the presence of dextran 500 (results not shown; the hydrodynamic diameters of dextran 4 and 500 were estimated to be 3.5 and 28 nm, respectively) (15, 16). The results suggested that Ply formed membrane pores with a functional diameter of 3.8–5.0 nm.

Sphingomyelin-binding Activity of Ply A and Sphingomyelin-specific Hemolysis—Erythrocytes from various mammalian species exhibited different susceptibilities to Ply, and the concentrations of Ply A and B for inducing 50% hemolysis were estimated to be 7, 50, 120, 4800, and 14,000 ng/ml for the erythrocytes from sheep, human, rabbit, dog, and horse, respectively (Fig. 6A). The results are consistent with those of Bernheimer and Avigad (12), and the susceptibility of erythrocytes to Ply may correlate with sphingomyelin content of the cell membrane (23). To clarify what component of Ply differentiates the mammalian erythrocytes, we assayed the binding of Ply A
Fig. 5. Ply-induced lysis of human erythrocytes in the presence of poly(ethylene glycol) of different molecular sizes. Human erythrocytes were incubated with Ply A and B at 25°C for 30 min in the absence (●) or presence of poly(ethylene glycol) 200 (○), 600 (●), 1000 (●), 2000 (●), 3000 (●), 4000 (●), 6000 (●), 8000 (●), or 10,000 (●).

Fig. 6. Hemolytic and binding activities of Ply to mammalian erythrocytes and sphingomyelin-specific inhibition of Ply-induced hemolysis. A, hemolytic activity of Ply toward the erythrocytes (3 × 10⁷ cells/ml) from sheep (○), human (●), rabbit (□), dog (■), and horse (△). Representative results from three independent experiments are illustrated. B, binding of Ply A to mammalian erythrocytes. Erythrocytes (6 × 10⁷ cells/ml) were incubated with or without Ply A (0.5 μg/ml) at 25°C for 30 min. Cell-bound Ply A was analyzed by Western immunoblot. RBC, red blood cells. C, inhibition of Ply-induced hemolysis by phospholipid-cholesterol liposomes. Human erythrocytes (3 × 10⁷ cells/ml) were incubated with Ply (final concentration of each component, 0.2 μg/ml) in the presence of the liposomes composed of sphingomyelin (○), phosphatidylcholine (●), phosphatidylethanolamine (■), phosphatidyserine (□), phosphatidylglycerol (△), or cardiolipin (△) and cholesterol.

and B to the cells. Ply A bound to the erythrocytes from sheep, human, and rabbit more efficiently than to those from dog and horse (Fig. 6B), whereas Ply B did not substantially bind to the mammalian erythrocytes (results not shown). The results suggested that Ply A determined the susceptibility of erythrocytes to Ply. To test the sphingomyelin specificity of Ply, we assayed the hemolytic activity of Ply in the presence of multilamellar liposomes composed of each of various phospholipids and cholesterol in a molar ratio of 1:1. Sphingomyelin-cholesterol liposomes inhibited Ply-induced hemolysis in a dose-dependent manner at 1–10 μM lipids, whereas the other liposomes revealed no inhibition at higher concentrations of up to 320 μM lipids (Fig. 6C). Thus Ply lysed erythrocytes in a sphingomyelin-dependent manner.

To demonstrate sphingomyelin-specific binding of Ply A, we assayed the binding of Ply A to various phospholipid-cholesterol liposomes. As a result, Ply A bound to sphingomyelin-cholesterol liposomes but not to the other liposomes composed of phosphatidylcholine, phosphatidyserine, phosphatidylglycerol, or phosphatidylethanolamine and cholesterol (Fig. 7A). Ply A caused CF leakage from sphingomyelin-cholesterol liposomes in concert with Ply B but not from the other liposomes (Fig. 7B). Neither Ply A nor B induced CF leakage from the sphingomyelin-cholesterol liposomes without their counterpart (results not shown). Ply A bound to the sphingomyelin membranes containing ≥30 mol% cholesterol, and Ply-induced CF release from sphingomyelin liposomes increased with increasing cholesterol content (Fig. 7, C and D). Furthermore, Ply A exhibited no sphingomyelinase activity (results not shown). Thus, Ply A is a sphingomyelin-binding protein that permeabilizes sphingomyelin-cholesterol membranes in concert with Ply B. In contrast, Ply B slightly bound to the liposomes without phospholipid specificity (results not shown).

Sequential Binding of Ply A and B to Sphingomyelin-Cholesterol Membranes and Assembly of the Two Components into a Transmembrane Pore Complex—When human erythrocytes were exposed to Ply A and B, optical density (OD) of the cells at 700 nm decreased after a time lag of 2 min and reached 10% of the initial level within 10 min (Fig. 8A). In contrast, OD of the cells at 700 nm did not decrease within 10 min upon exposure to either Ply A or B alone (Fig. 8A). To study sequential action of Ply A and B, human erythrocytes were incubated with Ply A or B and washed with TBS, and the washed cells were subsequently exposed to the counterpart. When human erythrocytes were exposed to Ply A and B in this sequence, OD of the cells at 700 nm rapidly decreased after a time lag of 1–2 min (Fig. 8A, PlyA→B). However, OD of human erythrocytes at 700 nm did not change when the cells were exposed to Ply B and subse-
A alone, Ply B alone, or Ply A and B, respectively, were added to the erythrocyte suspensions at time zero. PlyA° respectively, at 25 °C were incubated with either Ply A or B at 25 °C. A and B, respectively.

A complex. (Fig. 8 B the 700-kDa band when a higher concentration of Ply was used of the molecular size markers. CBB stain revealed formation of an SDS-stable high molecular weight complex was estimated to be ~700,000 by extrapolating the standard curves of the molecular size markers. CBB stain revealed formation of the 700-kDa band when a higher concentration of Ply was used (Fig. 8 B, lane PlyA+B). In contrast, when human erythrocytes were incubated with either Ply A or B alone, neither hemolysis nor formation of the 700-kDa band was detected (Fig. 8 C, lanes PlyA and PlyB). To study the sequential incorporation of Ply A and B into the 700-kDa complex, human erythrocytes were sequentially exposed to Ply A and B, and cell membranes were analyzed by SDS-PAGE and Western immunoblot using antisera against Ply A and B. When human erythrocytes were incubated with Ply A and B at 25 °C for 30 min, an SDS-stable high molecular weight band was detected by the Western immunoblot (Fig. 8 C, lane PlyA+B). The size of the high molecular weight complex was estimated to be ~700,000 by extrapolating the standard curves of the molecular size markers. CBB stain revealed formation of the 700-kDa band when a higher concentration of Ply was used (Fig. 8 B, lane PlyA+B). In contrast, when human erythrocytes were incubated with either Ply A or B alone, neither hemolysis nor formation of the 700-kDa band was detected (Fig. 8 C, lanes PlyA and PlyB). To study the sequential incorporation of Ply A and B into the 700-kDa complex, human erythrocytes were sequentially exposed to Ply A and B, and cell membranes were analyzed by SDS-PAGE and Western immunoblot using antisera against Ply A and B.}

**Fig. 8.** Sequential action of Ply A and B on human erythrocytes and assembly of the two components into a high molecular weight complex. A, change in OD at 700 nm of human erythrocytes (6 × 10⁷ cells/ml) upon exposure to Ply (final concentration of each of the components, 0.5 µg/ml) at 25 °C. B and C, complex formation by Ply. Human erythrocytes (6 × 10⁷ cells/ml) were incubated with Ply at 25 °C for 30 min, and cell-bound Ply was solubilized with 2% (w/v) SDS and subjected to SDS-PAGE (B) and Western immunoblot (C). Ply A and B were used at the final concentration of 5 or 0.5 µg/ml in the experiments of B and C, respectively. Protein bands were stained with CBB, PlyA, PlyB, and PlyA+B: Ply A alone, Ply B alone, or Ply A and B, respectively, were added to the erythrocyte suspensions at time zero. PlyA—B and Ply B—A: erythrocytes were incubated with either Ply A or B at 25 °C for 30 min and washed with TBS, and the washed cells were subsequently exposed to Ply B or A, respectively, at 25 °C for 30 min. Closed and open arrowheads indicate the positions of the high molecular weight band and the monomers of Ply A and B, respectively.

To study whether Ply A and B assemble into transmembrane pore complex(es) on human erythrocytes, cell-bound Ply was solubilized with 2% (w/v) SDS at 25 °C and subjected to SDS-PAGE and Western immunoblot using antisera against Ply A and B. When human erythrocytes were incubated with Ply A and B at 25 °C for 30 min, an SDS-stable high molecular weight band was detected by the Western immunoblot (Fig. 8 C, lane PlyA+B). The size of the high molecular weight complex was estimated to be ~700,000 by extrapolating the standard curves of the molecular size markers. CBB stain revealed formation of the 700-kDa band when a higher concentration of Ply was used (Fig. 8 B, lane PlyA+B). In contrast, when human erythrocytes were incubated with either Ply A or B alone, neither hemolysis nor formation of the 700-kDa band was detected (Fig. 8 C, lanes PlyA and PlyB). To study the sequential incorporation of Ply A and B into the 700-kDa complex, human erythrocytes were sequentially exposed to Ply A and B, and cell membranes were analyzed by SDS-PAGE and Western immunoblot. When human erythrocytes were exposed to Ply A and Ply B in this sequence, the 700-kDa complex was formed (Fig. 8, B and C, lane PlyA→B). However, when human erythrocytes were exposed to the two components in the reversed sequence, no high molecular weight complex was recovered in the fractions corresponding to 19–23% sucrose, whereas Ply A monomers were distributed in the fractions of 10–15% sucrose (Fig. 10, A and B). Thyloglobulin (669 kDa) was recovered in the fraction of 21% sucrose (Fig. 10, A and B). Electron microscopy showed the presence of a ring-shaped structure with outer and inner diameters of 14 and 7 nm, respectively, in the fractions of 19–23% sucrose (Fig. 10, C–E). Ring-shaped structures of the same dimensions were observed when Ply-treated sphingomyelin-cholesterol liposomes were solubilized with 2% SDS and subjected to electron microscopy (results not shown).
In this study, we purified Ply consisting of A (17 kDa) and B (59 kDa) components from the edible mushroom *P. ostreatus*. Our results suggest that the pleurotolysin (12 kDa) of Bernheimer and Avigad (12) is a proteolytic product from Ply A, and its hemolytic activity is caused by the cooperation with Ply B. Although the N-terminal amino acid sequence of Ply A has 93% identity with that of ostreolysin, they are distinct proteins possibly with different C-terminal parts on the basis of the following facts. (i) Ply A exhibits no hemolytic activity without Ply B (Fig. 3B). (ii) Ply-induced lysis of human erythrocytes is specifically inhibited by sphenomytlin (669 kDa). C-E, electron microscopy for the negatively stained pore complexes. Open arrowheads indicate the ring-shaped structures. Bars indicate 30 nm.

**DISCUSSION**

In this study, we purified Ply consisting of A (17 kDa) and B (59 kDa) components from the edible mushroom *P. ostreatus*. Our results suggest that the pleurotolysin (12 kDa) of Bernheimer and Avigad (12) is a proteolytic product from Ply A, and its hemolytic activity is caused by the cooperation with Ply B. Although the N-terminal amino acid sequence of Ply A has 93% identity with that of ostreolysin, they are distinct proteins possibly with different C-terminal parts on the basis of the following facts. (i) Ply A exhibits no hemolytic activity without Ply B (Fig. 3B). (ii) Ply-induced lysis of human erythrocytes is specifically inhibited by sphenomytlin (669 kDa). C-E, electron microscopy for the negatively stained pore complexes. Open arrowheads indicate the ring-shaped structures. Bars indicate 30 nm.

**REFERENCES**

1. Bernheimer, A. W., and Rudy, B. (1986) Biochim. Biophys. Acta 864, 123-141.
2. Bhakdi, S., and Tranum-Jensen, J. (1987) Rev. Physiol. Biochem. Pharmacol. 107, 147-225.
3. Memetna, G., Dalla Serra, M., Consani, M., Coraisla, M., Viero, G., Werner, S., Colin, D., Montei, H., and Prevost, G. (2003) FEBS Lett. 552, 54-60.
4. Montoya, M., and Gouaux, E. (2003) Biochim. Biophys. Acta 1609, 19-27.
5. Faulstich, H., Buehring, H. J., and Seitz, J. (1983) Biochemistry 22, 4574-4580.
6. Wilmsen, H. U., Faulstich, H., Ebbi, H., and Böheim, G. (1985) Eur. Biophys. J. 14, 189-200.
7. Lin, J. Y., Lin, Y. J., Chen, C. C., Wu, H. L., Shi, G. Y., and Jeng, T. W. (1974) Nature 253, 235-237.
8. Tomita, T., Ishikawa, D., Noguchi, T., Katayama, E., and Hashimoto, Y. (1998)
Pleurotolysin, a Novel Sphingomyelin-specific Two-component Cytolysin from the Edible Mushroom *Pleurotus ostreatus*, Assembles into a Transmembrane Pore Complex

Toshio Tomita, Kayoko Noguchi, Hitomi Mimuro, Fumio Ukaji, Kiyoshi Ito, Noriko Sugawara-Tomita and Yohichi Hashimoto

*J. Biol. Chem.* 2004, 279:26975-26982.
doi: 10.1074/jbc.M402676200 originally published online April 14, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M402676200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 31 references, 8 of which can be accessed free at http://www.jbc.org/content/279/26/26975.full.html#ref-list-1