**Pleurotus** and **Agrocybe** hemolysins, new proteins hypothetically involved in fungal fruiting

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**Abstract**

Novel hemolytic proteins, ostreolysin and aegerolysin, were purified from the fruiting bodies of the edible mushrooms **Pleurotus ostreatus** and **Agrocybe aegerita**. Both ostreolysin and aegerolysin have a molecular weight of about 16 kDa, have low isoelectric points of 5.0 and 4.85, are thermolabile, and hemolytic to bovine erythrocytes at nanomolar concentrations. Their activity is impaired by micromolar Hg2+ but not by membrane lipids and serum low-density lipoproteins (LDL). The sequence of respectively 50 and 10 N-terminal amino acid residues of ostreolysin and aegerolysin has been determined and found to be highly identical with a cDNA-derived amino acid sequence of putative Aa-Pri1 protein from the mushroom **A. aegerita**, Asp-hemolysin from **Aspergillus fumigatus**, and two bacterial hemolysin-like proteins expressed during sporulation. We found that ostreolysin is expressed during formation of primordia and fruiting bodies, which is in accord with previous finding that the Aa-Pri1 gene is specifically expressed during fruiting initiation. It is suggestive that the isolated hemolysins play an important role in initial phase of fungal fruiting. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** **Pleurotus ostreatus**; **Agrocybe aegerita**; Mushroom; Hemolytic protein; Fungal fructification

1. **Introduction**

So far, several cytolytic proteins have been isolated from different species of *Ascomycotina* and *Basidiomycotina* [1–10]. One of them is Asp-hemolysin from *Aspergillus fumigatus* [1], a 16-kDa protein with an isoelectric point of 4.0. Hemolytic activity of this protein is enhanced in the presence of Zn2+ or Co2+, and inhibited by iodine or Hg2+ [1]. Asp-hemolysin was found to bind with a high affinity to plasma lipoproteins, in particular low-density lipoproteins (LDL) [11,12]. The gene encoding Asp-hemolysin was cloned and sequenced [13], showing local sequence similarities (26.5% identity) with an LDL receptor precursor. Structural studies of fungal genes have shown that the cDNA-derived amino acid sequence of a putative Aa-Pri1 protein from the mushroom *Agrocybe aegerita* shows 39.1% identity with Asp-hemolysin [14]. The Aa-Pri1 gene was found to be specifically expressed during fruiting initiation, and suggested to encode an acidic 16-kDa protein [14].

Based on the high similarity reported for Asp-hemolysin and the putative Aa-Pri1 initiator, we addressed the question if Aa-Pri1 or related proteins could be detected as hemolysins during fruiting of two mushrooms, *A. aegerita* and *Pleurotus ostreatus*. In fact, *P. ostreatus* has already been reported to produce a hemolytic protein, pleurotolysin [10]. We found that both mushrooms in our study produce 16-kDa hemolysins, named aegerolysin and ostreolysin. N-terminal sequencing proved them to be nearly identical to the cDNA-derived amino acid sequence of the putative fruiting initiator Aa-Pri1 from *A. aegerita*. We found moreover that ostreolysin expression confers with development of primordia and fruiting bodies of *P. ostreatus*.

2. **Materials and methods**

2.1. **Strains and cultivation of mushrooms**

The presence and the hemolytic activity of ostreolysin and aegerolysin were monitored at different developmental
stages of mushrooms, which were cultivated according to Stamets and Chilton [15]. *P. ostreatus* (strain Plo 5) and *A. aegerita* (strain PAP2 98) were taken from the fungal collection of the Department of Wood Science and Technology, Biotechnical Faculty, University of Ljubljana, Slovenia. Mycelia of *P. ostreatus* were grown at 25 °C in dark on 90% pine wood sawdust and 10% wheat bran with 70% of moisture content, while mycelia of *A. aegerita* were grown under the same conditions on 90% poplar chips and 10% wheat bran with 70% of moisture content. After 4 weeks, growing mycelia were transferred for 4 days to 8–10 °C in dark, in order to induce formation of primordia. The cultivation process proceeded in a growth chamber with a constant temperature of 16 °C, 90% air humidity, less then 1000 ppm CO₂, and a 12:12 photoperiode (29.4 W/m²). Formation of primordia in *A. aegerita* was additionally induced by covering the surface of the mycelia with sterilized peat 15 days after the transfer to the growth chamber. After the primordia have developed, the humidity was decreased to 85–92%, and the CO₂ increased to 1500–2000 ppm.

Samples of the growing mycelia, primordia and mushroom tissues were homogenized and extracted with 50 mM TRIS-HCl, pH 8.5, and centrifuged for 10 min at 13,000 rpm and 4 °C. The supernatants were used for determination of protein contents, hemolytic activity, SDS-PAGE, and Western blotting analysis.

### 2.2. Isolation of hemolytic proteins

Hemolysins were isolated from fresh fruiting bodies of *P. ostreatus* or *A. aegerita* using identical procedures. Fresh mushrooms (300 g) were homogenized with 300 ml of 50 mM TRIS-HCl, pH 8.5. The homogenate was centrifuged twice for 30 min at 15,500 rpm. The resulting supernatant was fractionated with solid ammonium sulfate. The hemolytically active fraction, obtained between 35% and 65% saturated ammonium sulfate, was dissolved in 50 mM TRIS-HCl buffer, pH 8.5, and applied to a Sephadex G-50 column (BioRad, USA). The sequence similarity search was performed using the Fasta + Beauty algorithm against the NCBI nonredundant protein database [17,18]. Amino acid sequences were aligned by the ClustalW software [19]. Pfam Protein families [20] and ProDom [21] databases were searched for protein family classification of the isolated hemolysins.

### 2.3. Electrophoresis techniques

To estimate the molecular weight and to check the purity, the isolated proteins were dissolved in electrophoresis buffer containing SDS with or without 5% β-mercaptoethanol. The samples were analyzed with SDS-PAGE using a homogenous 20% acrylamide gel (Phast System, Pharmacia). Proteins were stained with Coomassie Blue.

The isoelectric focusing of the proteins was performed on a 0.2% acrylamide gel with a pH gradient of 3–9 (PhastGel™ IEF 3-9, Pharmacia).

All the electrophoresis techniques were performed according to the manufacturer’s protocols [16].

### 2.4. Protein sequencing and sequence analysis

The N-terminal amino acid sequence analysis of isolated hemolysins was performed on a Procise protein sequencing system 492A (PE Applied Biosystems, Foster city, CA, USA). The sequence similarity search was performed using the Fasta + Beauty algorithm against the NCBI nonredundant protein database [17,18]. Amino acid sequences were aligned by the ClustalW software [19]. Pfam Protein families [20] and ProDom [21] databases were searched for protein family classification of the isolated hemolysins.

### 2.5. Estimation of hemolytic activity

Hemolytic activity was measured using bovine erythrocytes as described previously [22]. Typically, 10–50 μl of hemolytic protein dissolved in 50 mM TRIS-HCl buffer, pH 8.5, were added into a cuvette containing 3.0 ml of bovine erythrocyte suspension in 0.13 M NaCl, 20 mM TRIS-HCl, pH 7.4, at 25 °C. The suspension had an apparent absorbance of 0.500 at a wavelength of 700 nm. Decrease of the apparent absorbance was recorded at 700 nm using a Shimadzu 2100 UV/VIS spectrophotometer to obtain the time course of hemolysis and t₅₀, the time required for 50% hemolysis. One hemolytic unit (HU) was defined as the amount of hemolysins that induced 50% lysis of bovine erythrocytes in 1 min.

To assay the inhibition of hemolytic activity by LDL, human plasma LDL (Sigma, USA) at a final concentration of 20 μg/ml, was added to 3 ml erythrocyte suspension, then 1 HU of ostreolysin or aegerolysin was added and the hemolysis was monitored.

The effect of membrane lipids on ostreolysin-induced hemolysis was studied using a competition method [23]. Four microliters of an ethanolic stock solution of phosphatidylserine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidyl-inositol, sphingomyelin, gangliosides, cerebrosides, or cholesterol were added to 3.0 ml of eryth-
rocyte suspension to achieve a final lipid concentration of about 10^{-7} M. The mixture was incubated for 1 min, then 1 HU of hemolysin was added, and the time course of hemolysis was monitored for 10 min. Sphingomyelin was from Avanti Polar Lipids (USA), all other lipids from Sigma. To study the effect of divalent cations, 1 HU of a hemolysin was added to 3 ml of an erythrocyte suspension supplemented with 0–500 μM ZnCl₂ or HgCl₂, and the hemolytic activity was measured.

Thermal stability of ostreolysin and aegerolysin, dissolved in 50 mM TRIS-HCl, pH 8.5, was estimated by heating the solution at 100 °C for 5 min before measuring hemolytic activity.

2.6. Preparation of anti-ostreolysin immune serum

Two rabbits (Department of Zootechnics, University of Ljubljana) were injected intracutaneously three times (3-week intervals) with 250 μl of 1.85 mg/ml ostreolysin in 50 mM TRIS-HCl, pH 8.5. The first inoculum contained a complete Freund’s adjuvant. After 2 months, sera were collected and kept at −20 °C until use.

2.7. Ostreolysin expression during the life cycle

At different developmental stages of P. ostreatus, ostreolysin production was assayed by monitoring hemolytic activity as described before, and by using Western blot analysis. For Western blotting, extracted proteins were separated with SDS-PAGE under reducing conditions as described above. Before electrophoresis, proteins in all the extracts were adjusted to a 0.15 mg/ml concentration by diluting them in 50 mM TRIS-HCl, pH 8.5. Proteins were blotted for 2 h to a 0.45-μm pore size polyvinylidene difluoride Immobilon™-P membrane (IPVH11510) (Millipore, USA), previously soaked in methanol for 10 min. The blotted membrane was fixed overnight in a 4% bovine serum albumin (BSA) solution in WASH buffer, composed of 50 mM NaCl, 1 mM EDTA and 10 mM TRIS-HCl, pH 7.4. The membrane was soaked and gently stirred for 2 h in 20 ml 4% BSA in WASH buffer combined with 200 μl of immune serum in order to allow binding of primary antibodies. After washing with WASH buffer, the membrane was treated for 2 h with goat anti-rabbit IgG-HRP secondary antibodies (Southern Biotechnology Associates, Inc., USA), previously diluted in 20 ml of WASH buffer containing 4% BSA. After unbound compounds had been washed out, the membrane was soaked to a peroxidase substrate, a mixture of 10 mg ethylcarbazole, 2.5 ml dimethylformamide, 47.5 ml 50 mM Na acetate, pH 5.0, and 1.2 ml 0.6% H₂O₂.

3. Results and discussion

We have isolated and partially characterized two new hemolytic proteins from mushrooms. They were purified by the fractionation of the crude extract with ammonium sulfate. The active fraction was separated by means of size-exclusion (Fig. 1A), ion exchange (Fig. 1B and C), and HPLC. The results of the purification procedure are summarized in Table 1.
Table 1
Purification procedure of hemolytic proteins from Pleurotus ostreatus (Po) and Agrocybe aegerita (Aa)

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Proteins (mg/ml)</th>
<th>V (ml)</th>
<th>Total activity (HU)</th>
<th>Specific activity (HU/mg)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>Po</td>
<td>Aa</td>
<td>Po</td>
<td>Aa</td>
<td>Po</td>
</tr>
<tr>
<td>Material</td>
<td>4.4</td>
<td>6.5</td>
<td>315</td>
<td>350</td>
<td>13,125</td>
</tr>
<tr>
<td>Precipitate with 35−65% saturated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ammonium sulfate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex G-50 active peak</td>
<td>0.7</td>
<td>0.97</td>
<td>69</td>
<td>85</td>
<td>8625</td>
</tr>
<tr>
<td>HighQ ion exchange—active peak</td>
<td>0.32</td>
<td>0.5</td>
<td>27</td>
<td>35</td>
<td>2250</td>
</tr>
<tr>
<td>ResourceQ ion exchange—active peak</td>
<td>0.4</td>
<td>0.55</td>
<td>20</td>
<td>30</td>
<td>2222</td>
</tr>
</tbody>
</table>

HU = hemolytic unit, or the amount of proteins that induced 50% lysis of bovine erythrocytes in one minute. V = volume of the samples.

SDS-PAGE analysis of purified ostreolysin revealed a single band at 17 kDa under reducing, and two bands at approximately 17 and 16 kDa under non-reducing conditions (Fig. 2A). An additional band could not be ascribed to the presence of impurities since (i) isoelectric focusing revealed only one band with an isoelectric point of 5.0 (Fig. 2B), (ii) reversed-phase HPLC analysis showed only one sharp peak (not shown), (iii) the amino-acid sequence analysis of a purified hemolysin revealed only one N-terminus. One possible explanation could be a partial intramolecular oxidation of cysteines during the isolation procedure, resulting in two conformational states of the protein. In the presence of β-mercaptoethanol eventual disulfide bridges may be reduced, and hence SDS-PAGE reveals a single band with a virtually higher molecular weight. Actually, in the putative Aa-Pr1 protein two cysteine residues could be translated from cDNA [14]. Aegerolysin however migrated as a single band of 17 kDa under reducing, and 16 kDa under non-reducing conditions (Fig. 2A). A single band of aegerolysin was isoelectric at pH 4.85 (Fig. 2B).

The sequence of respectively 50 and 10 N-terminal amino acid residues of ostreolysin and aegerolysin was determined. That of ostreolysin was 68.0% identical to the cDNA-derived hypothetical Aa-Pr1 amino acid sequence from A. aegerita [14] (Fig. 3). Both aegerolysin and ostreolysin lacked 8 N-terminal amino acid residues that had been suggested in the putative Aa-Pr1 protein. It is evident that this propeptide is post-translationally cleaved off as also found for P16 variants of hemolysin-like Cbm17.1 and Cbm17.2 proteins from C. bifermentans CH18 [24]. Aegerolysin appears slightly different from the cDNA-derived hypothetical Aa-Pr1 protein [14]; its N-terminal amino acid sequence (10 amino acids) was identical to ostreolysin, bearing an isoleucine at position 10, while in the cDNA-translated sequence leucine was determined at the position 10 (Fig. 3). We suggest that aegerolysin could exist in isoforms in different mushroom strains.

Besides to the Aa-Pr1 protein, the ostreolysin N-terminal fragment shows similarities to Asp-hemolysin from A. fumigatus [13] and P16 variants of hemolysin-like proteins Cbm17.1 and Cbm17.2 [24], with 46.9%, 43.9%, and 41.4% identity, respectively (Fig. 3). High similarity among these four proteins was also confirmed by the search performed in the Pfam Protein families database [20], which classified them as a unique family. An additional protein, the hypothetical PA0122 from Pseudomonas aeruginosa PA01 [25], was added to the family when the search was performed against the ProDom [21] database. Therein, Asp-hemolysin [13], the putative Aa-Pr1 protein [14], and hemolysin-like proteins from C. bifermentans CH18 [24] are described as acidic proteins with a molecular weight of about 16 kDa. Both ostreolysin and aegerolysin can be fitted into this family.

Ostreolysin and aegerolysin exert a sigmoidal course of hemolysis, which is dose-dependent. It occurs rapidly after the initial lag phase (inset of Fig. 4). Both proteins were active at nanomolar concentrations, ostreolysin being approximately threefold more potent (Fig. 4). Their hemolytic activity could be abolished by boiling the solution for 5 min. In contrast to sphingomyelin-inhibited pleurotolysin isolated from P. ostreatus [10], the hemolytic activity of ostreolysin was not inhibited by any of the tested lipids. Hemolysis was not enhanced by Zn²⁺ as reported for Asp-hemolysin [1]. It was however completely inhibited by 10 μM HgCl₂, and hemolytic activity was restored by the successive addition of 100 μM cysteine. This reflects the importance of cysteine residues in either the hemolytic
activity or protein structure. In contrast to Asp-hemolysin, none of the purified hemolysins could be inhibited by LDL.

Ostreolysin and aegerolysin bear a certain degree of structural similarity to Cbm17.1 and Cbm17.2, two weakly hemolytic proteins from *Clostridium bifermentans* [24]. This anaerobic bacterial strain is non-toxic to vertebrates but highly toxic to mosquito larvae. The authors proposed that Cbm17.1 and Cbm17.2 proteins might have a role in potentiating the activity of other insecticidal proteins found in *C. bifermentans* [24]. After the expression of cbm17.1 and cbm17.2 in a crystal-negative strain of *Bacillus thuringiensis*, Cbm17.1 and Cbm17.2 proteins were detected in the cell pellet during the late sporulation phase [24]. The authors suggested that the corresponding genes could be under the control of late exponential or early sporulation stage promotors. In this respect, it is notable that we have neither found any insecticidal activity of ostreolysin upon a feeding assay of *Drosophila melanogaster* (unpublished observation).

![Alignment of ostreolysin and aegerolysin amino acid sequences and structurally similar proteins](image1)

![Hemolytic activity of ostreolysin and aegerolysin](image2)

S. Berne et al. / Biochimica et Biophysica Acta 1570 (2002) 153–159

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**Fig. 3.** Alignment of ostreolysin and aegerolysin amino acid sequences and structurally similar proteins. Aa-Pr1, hemolysin from Agrocybe aegerita [14]; Asp-hemolysin, hemolysin from *A. fumigatus* [13]; Cbm17.2 and Cbm17.1, putative hemolysins from *Clostridium bifermentans* [24]. The alignment of amino acid sequences was performed using the ClustalW software [19]. The numbers in parentheses denote the amino acid residue ending the line.

2.4. **Fig. 4.** Hemolytic activity of ostreolysin and aegerolysin. Dependence of the rate of hemolysis, 1/t₅₀, on concentration of ostreolysin (○) and aegerolysin (●). Molar concentrations are calculated assuming a $M_W$ of 16 kDa for both proteins. Inset: Time course of hemolysis induced by ostreolysin (dotted line) and aegerolysin (solid line), both 8.5 μg/ml (531 nM); $A_{700}$ = apparent absorbance at a w.l. of 700 nm. For details, see Materials and methods.
Northern blot analysis of total cellular RNA, extracted at different developmental stages of *A. aegerita*, revealed the presence of a *Aa-Pri1* transcript at the stage of primordia and immature fruiting bodies only [14]. The putative *Aa-Pri1* protein has been suggested to play a specific role during the fruiting initiation of the mushroom. It was suggested to bind with specific membrane receptors in the hyphae, allowing their aggregation and compaction to form the primordia. As compared to the *Aa-Pri1* gene transcription [14], monitoring of hemolytic activity and the Western blots of ostreolysin at different developmental stages of *P. ostreatus* revealed very similar temporal patterns of synthesis (inset of Fig. 5). Specifically, neither hemolysis nor ostreolysin production were detected during the mycelial growth but appeared during formation of primordia (Fig. 5). Hemolytic activity sprouted remarkably in developing fruit bodies but started to decline by their maturing. Monitoring of hemolytic activity of the *A. aegerita* extracts revealed the same pattern (not shown). The decreased hemolytic activity found by maturation of the grown mushrooms may be interpreted in terms of a decreased rate of the transcription as already reported for the *Aa-Pri1* [14].

In conclusion, several genes specifically expressed during fruiting initiation have been recently cloned and sequenced from cultivated edible mushrooms [26,27]. The *Aa-Pri1* gene has been reported to be specifically expressed during fruiting initiation of the mushroom *A. aegerita* [14]. Our study provides evidence that the isolated hemolysins aegerolysin and ostreolysin are very likely identical to the putative *Aa-Pri1* protein, which has been suggested to be involved in fungal fruiting. Moreover, these hemolysins appear to be homologous to ascomycetal Asp-hemolysin and hemolysin-like proteins from *C. bifermentans* expressed during sporulation. It however remains to be elucidated if these homologous proteins play a role in common to ascomycetal and basidiomycetal fungi, or bacteria.

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References


