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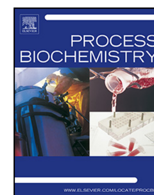

Short communication

# An acid-tolerant lectin coupled with high $Hg^{2+}$ potentiated hemagglutination enhancing property purified from *Amanita hemibapha* var. *ochracea*

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ABSTRACT

A 37.4 kDa acid tolerant lectin was isolated and purified from dried fruiting bodies of *Amanita hemibapha* var. *ochracea* designated as AHL. The lectin was not adsorbed on DEAE-cellulose, but rather adsorbed on S-Sepharose and subjected to gel filtration by fast protein liquid chromatography on Superdex 75. The purified lectin was immune from inhibition activities of metal ions. More over, AHL exhibited high agglutination activity on rabbit erythrocytes with accelerating $Hg^{2+}$ ions concentration. Partial peptide sequence analysis (VSNNLLTGPKVVR) of this lectin showed relative similarity to phosphoenolpyruvate carboxykinase [ATP]-like protein as predicted from *Fragaria vesca* subsp. *Vesca*. Interestingly, AHL displayed a strong affinity toward α-Lactose, making our study the first report associating *Amanita* species' lectin specificity for α-Lactose to the best of our knowledge.

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## 1. Introduction

Lectins have been purified from living organisms including animals, plants, fungi, bacteria and viruses. Lectins have also been purified from a number of mushroom species [1–5]. Nonetheless, lectins from mushrooms have received appreciable level of attention in the last two decades. In view of accumulating evidence, lectins from mushrooms execute variety of functions such as cell attachment, migration and invasion [6]. Further studies also reveal that mushroom lectins exhibit unique specificities for carbohydrates and biological activities, hence, endowing mushroom lectins with significant potentials in pharmacological and biotechnological applications [7]. Additionally, the biological activities of mushroom lectins are demonstrated in their applications in histochemical or as diagnostic reagents, especially for investigating the changes occurring on the cell membranes [8,9]. Most of the lectins characterized from mushrooms, have been shown to possess biological functions amongst which includes anti-proliferative activity against human cancer cell lines [5]. For instance; *Agaricus bisporus* lectin, showed potent antiproliferative activity against human colon cancer cell lines HT29 and breast cancer cell lines (MCF-7) [10]. *Volvariella volvacea* lectin showed antitumor activity to sarcoma S-180 cells [11], while *Tricholoma mongolicum* lectin showed anti-tumor on mouse mastocytoma P 815 cells *in vitro* and sarcoma S-180 cells *in vivo* [12]. Additionally *Grifola frondasa* lectin was also found to be cytotoxic to HeLa cell lines [13] just to mention the few. More so, some mushroom lectins expressed other potential activities such as immunoenhancing and antimicrobial activities [11,14], hemolytic activity on erythrocytes of human and numerous animal species [15], mitogenic activity toward spleen cells [16,17], hypotensive and insecticidal effects [18] as well as inhibitory activity toward HIV-1 reverse transcriptase [20].

In view of the great diversity of mushrooms lectins coupled with their wide medicinal attributes, we deemed it necessary to devote this study to further exploit the biochemical and biological properties of *Amanita hemibapha* var. *ochracea* lectin.

## 2. Materials and methods

### 2.1. Purification of lectin

Dried fruiting bodies of *A. hemibapha* var. *ochracea* were sampled from Sichuan province in China, and subsequently homogenized in

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distilled water (2.5 ml/g) using a Waring blender. It was then stored at 4 °C overnight before centrifugation. Ammonium sulfate precipitation was carried out by adding $(NH_4)_2SO_4$ to the supernatant up to 80% saturation to precipitate proteins. After centrifugation, the precipitated proteins were dissolved in distilled water and dialyzed to remove $(NH_4)_2SO_4$. NaAc–HAc buffer (pH 5.6, 1 M) was then added to the solution, until the concentration of NaAc reached 10 mM. The supernatant was subjected to ion exchange chromatography on a column of DEAE-cellulose (Sigma) in 10 mM NaAc–HAc buffer (pH 5.6). *A. hemibapha* var. *ochracea* lectin (AHL) was in the unadsorbed fraction (D1) washed with the same buffer, and the rest of proteins adsorbed on the column were eluted with 1 M NaCl in the buffer as a fraction D2 without AHL activity. Fraction D1 with AHL activity was dialyzed and subsequently chromatographed on a 2.5 × 10 cm of S-Sepharose (Sigma) in 10 mM NaAc–HAc buffer (pH 3.6). After removal of unadsorbed proteins (fraction S1), adsorbed proteins were eluted with a linear concentration gradient (0–0.5 M) of NaCl and 1 M NaCl in 10 mM NaAc–HAc buffer (pH 3.6). The peak (S2) with AHL activity was then further purified on a Superdex 75 HR 10/30 column (GE health) in 0.15 M $NH_4HCO_3$ buffer (pH 8.5). The peak (SU1) obtained represented purified lectin.

### 2.2. Molecular mass determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and by FPLC-gel filtration

The purified lectin was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) for molecular mass determination in accordance with the procedure of Laemmli and Favre [21]. Gel filtration on FPLC-Superdex 75 column, which had been calibrated with molecular mass markers (Amersham Biosciences), was conducted to determine the molecular mass of lectin.

### 2.3. Assay for lectin (hemagglutinating) activity

A serial two fold dilution of the lectin solution was mixed with 25 μl of a 2% suspension of rabbit erythrocytes in phosphate-buffered saline (pH 7.2) at 20 °C in microtiter U-plates (25 μl). The results were observed after approximately 1 h when the blank had fully sedimented. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was reckoned as one hemagglutination unit. Specific activity is the number of hemagglutination units/per mg protein [22].

### 2.4. Inhibition of lectin-induced hemagglutination by carbohydrates

The hemagglutinating inhibition tests to investigate the inhibition of lectin-induced hemagglutination by various carbohydrates were performed in a manner analogous to the hemagglutination test. Serial two-fold dilutions of test sugar samples were prepared in phosphate-buffer saline (PBS), pH 7.4. Each dilution was mixed with an equal volume (25 μl) of a solution of the agglutinin with 16 hemagglutination units. The mixture was allowed to stand for 30 min at 4 °C before mixing with 50 μl of 2% rabbit erythrocyte suspension. The minimum concentration of the test sugar in the final reaction mixture, which completely inhibited 8 hemagglutination units of the lectin, was calculated [23].

The following sugars were used; D-glucose, D-galactose, N-acetyl-D-galacosamine (GalNAc), N-acetyl-D-glucosamine, mannose, xylose, fucose, raffinose, arabinose, rhamnose, and lactose, were investigated.

### 2.5. Effects of various metal ions on hemagglutination activity

The effects of different metal ions on the activity of the AHL were studied. 25 μl dialyzed lectin solution was mixed with metal ion in the respective concentrations: 25 μl; 20 mM, 10 mM, 5 mM, 2.5 mM The metal ions used were $Ca^{2+}$, $K^{+}$, $Mn^{2+}$, $Zn^{2+}$, $Mg^{2+}$, $Cd^{2+}$, $Al^{3+}$, $Pb^{2+}$, $Cu^{2+}$, $Fe^{3+}$, $Fe^{2+}$. AHL was incubated with metal ion at 4 °C for 1 h in the fridge and an aliquot was used for the activity measurement. With 16 hemagglutination units, a serial two-fold dilution of the mixtures (diluted with 0.15 M NaCl) (25 μl) were mixed with 25 μl of 2% suspension of rabbit red blood cells in 0.15 M NaCl at room temperature. The hemagglutinating results were read after 1 h when the blank had fully sedimented. A control assay of the activity was done without metal ions in 0.15 M NaCl solution and the resulting activity was taken as 100%.

### 2.6. Heat and pH stability

The heat and pH stability of the selected lectin was examined according to the modified method of Kobayashi et al. [24]. Heat stability was determined by heating aliquots of the lectin (1 mg/ml lectin solution in PBS) for 30 min at various temperatures of 10, 20, 30, 40, 50, 55, 65, 70, and 100 °C. Then the heated aliquots, were cooled rapidly on ice, centrifuged to remove any precipitate, and assayed for hemagglutination activity in comparison with a control sample. Results were expressed by calculating the percentage of hemagglutination shown by the heated aliquots (titration value) compared with the control sample representing 100%. For the pH dependency, the lectin sample incubated with 12.5–100 mM HCl and NaOH for 60 min at room temperature, then equivalent alkali and acid was used to neutralize, and assayed for hemagglutination activity in comparison with a control sample. Results were calculated by expressing the titration values of the lectin as percentages of the titration value of the control.

### 2.7. Analysis of partial amino acid sequence

Single band of purified lectin from SDS–PAGE was cut off and sent to National Centre of Biomedical Analysis (Beijing) for analysis of amino acid sequence by Q-TOF.

### 2.8. Assay for HIV-1 reverse transcriptase inhibitory activity

The assay was carried out according to instructions supplied with the assay kit from Boehringer Mannheim (Germany). The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly (A) oligo (dT) 15. The digoxigenin and biotin-labeled nucleotides in an optimized ratio were incorporated into one of the same DNA molecule, which was freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol. The absorbance of the samples at 405 nm can be determined using a microtiter plate (ELISA) reader and was directly correlated with the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of the lectin was calculated as percent inhibition compared to a control without the protein [25].

### 2.9. Cytotoxicity test against cancer cell lines

The antiproliferative activity of the purified protein was determined as follows. The cell lines HepG2 and MCF-7 were maintained in Dulbecco Modified Eagles' Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/l streptomycin and 100 IU/ml penicillin, at 37 °C in a humidified atmosphere of 5% (v/v) $CO_2$. Cells ($1 \times 10^4$) in their exponential growth phase were seeded in each well of a 96-well culture plate (Nunc, Denmark) and incubated 12 h before addition of the purified lectin. Incubation was carried out for another 48 h. Radioactive precursor, 1 μCi ([methyl-H] thymidine,

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