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N-acetylated α-linked acidic dipeptidase is identified as an antigen of *Histoplasma capsulatum*

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ABSTRACT

Histoplasmosis, one of the most important mycoses, needs to be diagnosed rapidly and accurately. The main method used to diagnose histoplasmosis is serological detection of antibodies to the *Histoplasma capsulatum* H and M antigens. Several other protein antigens have been reported in *H. capsulatum*; however, they have not been used for diagnosis. In this study, we explored novel antigens that were detected during *H. capsulatum* infection. We obtained a protein mixture from *H. capsulatum* yeast cells after vigorous mixing in a 0.1% Triton X-100 solution. From the resultant pool, we detected nine spots that reacted with sera from patients with histoplasmosis and identified eight seroactive proteins with mass spectrometry. The seroactive proteins were purified, and their antigenicities were tested with an enzyme-linked immunosorbent assay (ELISA). ELISA revealed that the titer of the patients' sera to *N*-acetylated α-linked acidic dipeptidase was significantly higher than those of healthy volunteers ($P < 0.01$). These data indicate that *N*-acetylated α-linked acidic dipeptidase of *H. capsulatum* is recognized as a major antigen during histoplasmosis.

1. Introduction

Human histoplasmosis is caused by *Histoplasma capsulatum*, a dimorphic fungus that is widely distributed throughout most of the world, particularly in the endemic areas such as the Ohio–Mississippi river valley. However, it has never been isolated from the Japanese environment [1]. Therefore, histoplasmosis has been classified as an imported mycosis in Japan. In the United States, approximately half a million cases of histoplasmosis are reported every year [2,3]. In Japan, the number of patients with histoplasmosis is small, but it is increasing [http://clinical-r.pf.chiba-u.jp/mycosis/01.html (in Japanese)] [4,5].

Serological tests are helpful for diagnosing histoplasmosis. The M and H antigens, which are the major *H. capsulatum* antigens, have homology to catalase and β-glucosidase, respectively [6,7]. The filtrate of *H. capsulatum* mycelial culture containing M and H antigens is used for diagnosis [8]. In addition, several seroactive proteins have been reported; however, these antigens are limited and have not been used in the diagnosis of histoplasmosis.

In this study, we explored novel antigens from *H. capsulatum* and investigated the reactions of the identified proteins with sera from patients with histoplasmosis.

2. Materials and methods

Fungi, bacteria, and growth conditions — The *H. capsulatum* CDC105 strain [9] was transformed into the yeast form and provided by the culture collection of the Medical Mycology Research Center, Chiba University. Yeast cells were grown on brain–heart infusion (BHI) agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) that was supplemented with 0.1% yeast extract (Becton, Dickinson and Company) and 1% glucose (modified-BHI) at 37 °C. *Escherichia coli* DH5α and BL21(DE3) were used for genetic

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manipulations and protein preparations. *E. coli* were routinely grown in lysogeny broth containing the appropriate antibiotics.

Preparation of the antigenic proteins from H. capsulatum yeast – *H. capsulatum* CDC105 was inoculated and grown on modified-BHI agar slants in the yeast form at 37 °C for 3 weeks. The cells were collected and suspended in sterilized water (1 mL per slant). The suspension was vigorously mixed for 1 min and then centrifuged. After collecting the supernatant, the yeast cells were washed three times with sterilized water and then suspended in sterilized water containing 0.1% Triton X-100. The suspension was vigorously mixed for 1 min. The supernatant was filtrated, and the proteins in the supernatant were then precipitated with deoxycholic acid (final concentration, 0.01%) and trichloroacetic acid (final concentration, 6.1%). The precipitant was washed with ethanol and dissolved in sample rehydration buffer (Life Technologies Corporation, Grand Island, NY, USA) for the isoelectronic focusing (IEF) described below.

Two-dimensional electrophoresis (2DE) – Next, 2DE was performed with the ZOOM IPGRunner System (Life Technologies Corporation). Approximately 500 μg of protein was applied to a ZOOM strip-pH 3–10 NL (Life Technologies Corporation), and IEF was performed as per manufacturer' instruction. After IEF, 2DE was performed with a Novex 4%–15% Bis–Tris gel (Life Technologies Corporation) according to the manufacturer's protocol. Proteins that were developed on the gel were visualized by staining them with Coomassie Brilliant Blue R-250 (CBB) (Quick-CBB; Wako Pure Chemical Industries, Ltd., Osaka, Japan), or they were transferred onto a polyvinylidene fluoride membrane. After the transfer step, immunodetection was performed with the procedure described in the *Dot-blot analysis* section.

Mass spectrometry – The spots of the proteins that reacted with the sera from the patients were excised from the gel stained with CBB. After destaining with the 50 mM NH_4HCO_3/50% methanol solution, washing with acetonitrile and drying the excised pieces, the proteins were digested with trypsin (Trypsin Gold; Promega Corporation, Madison, WI, USA) at 37 °C for 10–14 h. The digested peptides were extracted with 0.1% trifluoroacetic acid/50% acetonitrile solution and 0.1% trifluoroacetic acid/80% acetonitrile solutions. After evaporation and concentration, the peptides were separated by liquid chromatography and analyzed with a matrix-assisted laser desorption ionization–tandem time-of-flight mass spectrometer (4700 MALDI-TOF/TOF Analyzer; Life Technologies Corporation). To identify the proteins from the *H. capsulatum* NAm1predicted protein set (http://www.broad.mit.edu/annotation/genome/histoplasma_capsulatum/MultiHome.html), the mass spectrometry data were analyzed with Mascot MS/MS Ions Search (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS, Matrix Science Ltd., London, UK).

RNA preparation from H. capsulatum yeast and cloning of the genes encoding the seroactive proteins – We used an ISOGEN RNA Isolation Kit (Nippon Gene Co. Ltd., Tokyo, Japan) to prepare the total RNA from *H. capsulatum*. The yeast cells of *H. capsulatum* CDC105 were directly suspended in 1 mL of ISOGEN solution. The *H. capsulatum* yeast cells were crushed with approximately 0.5 mg of 0.5-mm-diameter glass beads (Yasui Kikai Corporation, Osaka, Japan) with the Multi-Beads Shocker (Yasui Kikai Corporation). The following RNA isolation steps were performed according to the manufacturer's instructions.

The genes encoding the seroactive proteins were amplified from the total RNA. The primers that were used are listed in Table S1. The amplified fragments were cloned into pQE80L vector (QIAGEN GmbH, Hilden, Germany) in order to be expressed as His-tagged proteins.

Production and purification of the recombinant proteins – The constructed plasmids described above were transformed into *E. coli* DH5α or BL21(DE3). The protein production was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside. The cultivated organisms were collected by centrifugation and suspended in Buffer A (50 mM Tris–HCl, 500 mM NaCl, and 20 mM imidazole, pH 7.5) and disrupted with the Multi-Beads Shocker or BIORUPTOR (Cosmo Bio Co., Ltd., Tokyo, Japan). The homogenate was separated by centrifugation, and the pellet containing the inclusion body was then washed with Buffer A and gently suspended in Buffer A that was supplemented with 8 M of urea (Buffer A w/urea). After centrifugation, the resulting supernatant containing the solubilized recombinant protein was transferred to the slurry of Ni-Sepharose 6 Fast Flow (GE Healthcare UK Ltd., Buckinghamshire, UK) and prewashed with Buffer A w/urea. Binding to the beads was performed at room temperature for 15 min. The slurry was washed with Buffer A w/urea and then eluted two times with Buffer B (50 mM Tris–HCl, 500 mM NaCl, and 500 mM imidazole, pH 7.5) that was supplemented with 8 M of urea (Buffer B w/urea). The resulting solutions were used in the assays described below.

ELISA – The recombinant proteins, except for heat-shock protein (Hsp) 60 and 70, were coated onto the wells of a Nunc-immuno MaxiSorp plate (Thermo Fisher Scientific Inc.). Due to the high background that occurred because of unknown reasons, Hsp60 and Hsp70 were coated onto the wells of a PolySorp plate (Thermo Fisher Scientific Inc.) instead of a MaxiSorp plate. In total, 200 ng [H antigen, M antigen, *N*-acetylated α-linked acidic dipeptidase (NAALADase; residues 385–713), protein disulfide isomerase, Hsp60, and Hsp70], 400 ng (catalase P, aconitase, and ATP synthase β subunit), or 1600 ng (dihydrolipoamide dehydrogenase) of the purified protein or Buffer B that was supplemented with urea, which was used as a control, was diluted with 200 μL of 0.05 M of carbonate buffer (pH 9.6) and coated on 96-well plates. After washing three times with TBS, the wells were blocked with Protein-Free Blocking Buffer at 25 °C for 1 h. These wells were washed three times with TBS-T with the ImmunoWash Model 1575 (Bio-Rad Laboratories, Inc.), which was followed by incubation with 100 μL of TBS containing 1% human serum at 25 °C for 2 h. Thereafter, the wells were washed three times with TBS-T with the ImmunoWash Model 1575, and 200 μL of 0.1 μg/mL Protein L-HRP in TBS was added to each well. The plates were incubated at 37 °C for 1 h and washed three times with TBS-T with the ImmunoWash Model 1575. For detection, the HRP substrate (TMB Peroxidase EIA Substrate Kit; Bio-Rad Laboratories, Inc.) was used according to the manufacturer's instructions. After the HRP reaction, the reaction was stopped by the addition of stop solution, and the plates were read at an absorbance at 450 nm by a Microplate Reader Model 550 (Bio-Rad Laboratories, Inc.). The data from the patients and healthy volunteer groups were analyzed using the Mann–Whitney U-test. To determine the cutoff, specificity, and sensitivity, EZR software was used [10].

Sera from patients with histoplasmosis or healthy volunteers – In the dot blot assay, we examined two sera samples from the healthy volunteers and four sera samples from the patients with histoplasmosis. We examined the sera from 22 healthy volunteers and 9 patients with histoplasmosis with ELISA. The clinical statuses and results of the diagnoses of the patients with histoplasmosis are shown in Table S2. We received approval to use the sera for this study from the ethics committee of the Medical Mycology Research Center, Chiba University, and we obtained informed consent from the volunteers.

3. Results

Extraction of the antigenic proteins from H. capsulatum yeast – To explore the antigenic proteins from *H. capsulatum*, we prepared a pool of proteins that contained the antigenic proteins. In the course

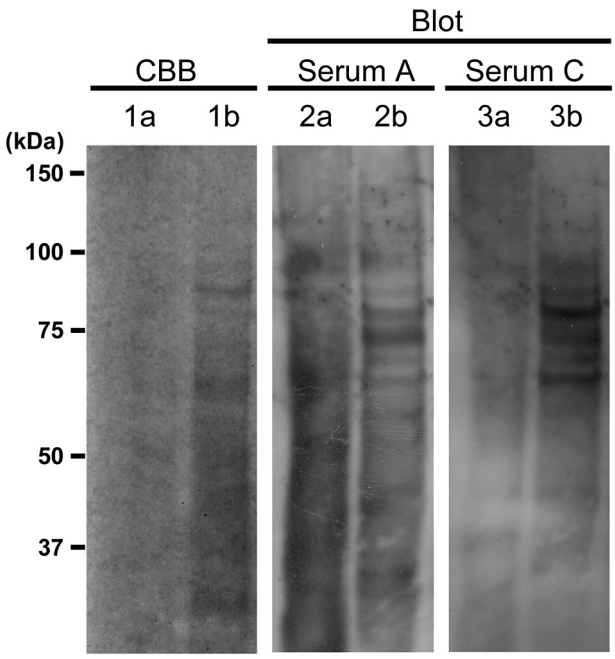

Fig. 1. Extracted proteins from *Histoplasma capsulatum* yeast (lanes 1a and b) and antigens that were reactive against the patient sera (lanes 2a, 2b, 3a, and 3b). Each "a" lane and each "b" lane contain the extract mixed with water or 0.1% Triton X-100, respectively. The proteins of lanes 1a and b were visualized by Coomassie Brilliant Blue (CBB) staining. Lanes 2 ab and lanes 3 ab were reacted with Serum A and Serum C, respectively. These original scanned images are shown in Fig. S4.

of *H. capsulatum* infection, this fungus propagates and persists in the form of yeast in the host. Therefore, the yeast cells of *H. capsulatum* were sampled for the preparation. After the extraction and vigorous mixing in water, obvious bands were not detected with CBB staining (Fig. 1, lane 1a). However, several proteins (50–100 kDa) of *H. capsulatum* yeast were detected after extraction and vigorous mixing in 0.1% Triton X-100 solution (Fig. 1, lane 1b), and several bands were visualized in the patient sera (Fig. 1, lanes 2b and 3b). We used the pool of proteins that were extracted in the 0.1% Triton X-100 solution for the following experiments.

Identification of the proteins that reacted with the sera from patients with histoplasmosis – The protein mixture was developed by 2DE, and the proteins were visualized with CBB staining (Fig. 2A). To determine the seroactive proteins, we performed immunoblot analyses on the sera from patients with histoplasmosis (Fig. 2B). As shown in Fig. 2, nine spots were visualized with immunoblotting. These seroactive proteins were cut out from the CBB-stained gel, extracted from the gel pieces, and then subjected to mass spectrometry analyses. We identified the proteins from these spots as the following: Hsp60, aconitase (also known as aconitate hydratase), catalase P, dihydrolipoamide dehydrogenase, protein disulfide isomerase, the ATP synthase β subunit, Hsp70, and NAALADase (Table 1). The M and H antigens, which are the major antigens of *H. capsulatum*, were not included in these spots.

Production of the seroactive proteins in E. coli – The genes encoding the seroactive proteins were cloned into the pQE80L vector for the production of His-tagged proteins. The seroactive proteins, except for NAALADase, were highly expressed in *E. coli*. The partial fragment of NAALADase (residues 385–713) was expressed, and the protein was used in the following experiments.

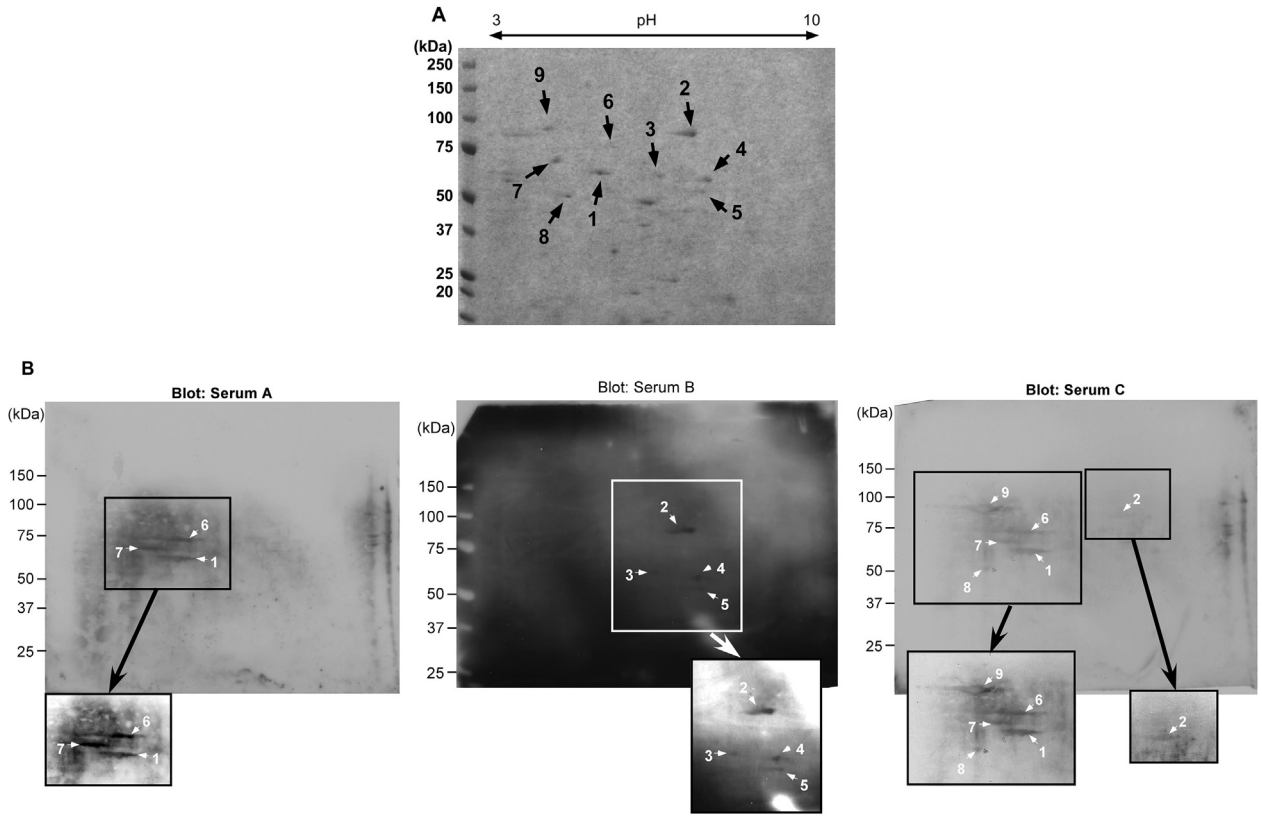

Fig. 2. The extracted *Histoplasma capsulatum* proteins that were visualized with CBB staining (*A*) on two-dimensional gels and the proteins that were recognized by the patients' sera (*B*). The numbers of the arrows in *A* correspond to the numbering of arrowheads in *B* and Table 1. The original image of the gel stained with CBB shown in Fig. S5. Small images in *B* show spots after contrast adjustment.

Table 1
The summary of seroactive proteins identified by Mascot MS/MS Ions Search.

Spot no.	Putative protein[a]	Locus[b]	Predicted molecular mass (kDa) and pI[c]	Calculated molecular weight (kDa)[d]
1	Heat shock protein 60	HCAG_06961	61.8/5.58	63.5
2, 3	Aconitate hydratase	HCAG_05266	85.2/6.74	90.0[e]
4	Catalase P	HCAG_08064	57.3/6.66	59.6
5	Dihydrolipoamide dehydrogenase	HCAG_08825	55.2/8.45	49.2
6	Heat shock protein 70	HCAG_01398	70.7/5.05	78.9
7	Protein disulfide isomerase	HCAG_03630	74.9/5.41	70.6
8	ATP synthase β chain	HCAG_06944	56.0/5.33	50.3
9	NAALADase	HCAG_05749	77.3/4.71	95.2

[a] Based on the search results using the set of predicted proteins of *H. capsulatum*.
[b] The locus tags from the *Histoplasma capsulatum* database of Broad Institute (http://www.broadinstitute.org/annotation/genome/histoplasma_capsulatum/MultiHome.html).
[c] Based on the amino acid sequence analysis using ExPASy Compute pI/MW tool (http://ca.expasy.org/tools/pi_tool.html).
[d] Calculated from electrophoretic mobility compared with standard marker.
[e] Corresponding to spot No. 2.

Because these expressed proteins formed inclusion bodies, urea was supplemented in the buffer that was used for the purification. The proteins that were solubilized by urea were purified on Ni Sepharose beads (Fig. S1).

The patient sera exhibited significantly higher titer to recombinant NAALADase *in the ELISA* — ELISA has been used as a rapid diagnostic technique. As shown in Fig. 3, the plates coated with the M and H antigens exhibited statistically significant differences between the IgG titer of the histoplasmosis group and that of the healthy group. Similarly, the plates coated with NAALADase exhibited statistically significant differences between the IgG titer of the histoplasmosis group and that of the healthy group (Fig. 3). When the other purified proteins were coated onto the plates that were used in ELISA, we could not detect significant differences between these groups, although some of the patients' sera exhibited high titers to the recombinant proteins, such as catalase P (Fig. 3). These results suggested that NAALADase of *H. capsulatum* were recognized as a major antigen during *H. capsulatum* infection.

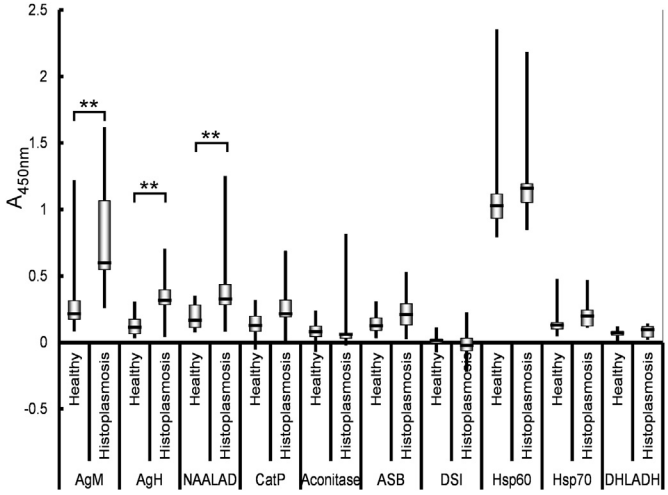

Fig. 3. The titers against the recombinant antigens, especially the M antigen, H antigen, NAALADase, and catalase P in the patient sera were higher than the titers in the healthy volunteer sera. The ordinate axis indicates the background-subtracted absorbance at 450 nm. In this experiment, the sera from 22 healthy volunteers (Sera a–v shown in Fig. S3) and 9 patients with histoplasmosis (Sera A–I in Fig. S3) were used. The upper end of the line indicates the maximum, while the lower end of the line indicates the minimum. The top of the box indicates the upper quartile, and the bottom of the box indicates the lower quartile. The horizontal black bar indicates the median. **: $P < 0.01$, Mann–Whitney U-test. AgM: M antigen, AgH: H antigen, NAALAD: NAALADase, CatP: catalase P, ASB: ATP synthase β chain, DSI: protein disulfide isomerase, DHLADH: dihydrolipoamide dehydrogenase.

4. Discussion

In this study, we identified NAALADase as a novel antigen of *H. capsulatum*. We expected that the M and H antigens would also be identified as seroactive proteins because they are well-known antigens and because the M antigen is expressed on the surface of yeast cells [11,12]. However, these antigens were not included in the spots. The reason might have been due to the procedure that was used to prepare the extract: Because the yeast cells were washed with water four times in our procedure, the secreted proteins that were not associated with the cell walls and membranes were lost.

We identified the protein in a spot as NAALADase that was approximately 35% identical to human NAALADase II (Fig. S2). These peptidases are widely known neuropeptidases that are expressed in the central nervous system of mammals [13] and found in a large number of fungi. However, the function in fungi remains unknown. In ELISA using recombinant NAALADase, the titer of the group of patients with histoplasmosis was significantly higher than that of healthy volunteers ($P < 0.01$), suggesting that NAALADase is recognized as a major antigen during histoplasmosis, like the H and M antigens. Because the epitopes of NAALADase and the cross-reactivity to sera from other fungal infections remain unknown, further studies are required to evaluate their recognition by the host and the specificity to histoplasmosis.

In summary, NAALADase was identified as an antigen in *H. capsulatum* infection, and the titer against NAALADase was significantly increased in the patient sera. Although further examinations are underway to clarify the utility of NAALADase, the antigen might be useful in the serological detection of histoplasmosis.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.129.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.129.

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