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Immunocytochemical localization of Ca^{2+}-dependent protease from *Allomyces arbuscula*

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Abstract

The Ca^{2+}-dependent protease antisera and the purified specific antibodies from *Allomyces arbuscula* have shown very specific recognition when blotted against the total protein extract or the purified 43–40 kDa Ca^{2+}-dependent protease from this aquatic fungus. By immunoblotting and immunofluorescence techniques using specific antibodies, we have shown that the enzyme activity is developmentally regulated and is related to the presence of antigen and not to any specific inhibitor. The immunofluorescence was absent in zoospores but appeared in polarized forms in germinating spores. In elongating hyphae the protease was mainly localized along the cytoplasmic membrane and in the cytoplasm, with predominance at the apex.

Key words: Aquatic fungus; Ca^{2+}-dependent protease; Cellular localization

1. Introduction

Proteolysis is one of the most important biological reactions in the cell. There are two types of proteases involved, one in the degradation of proteins into peptides and amino acids, and the other in the processing, through limited proteolysis, of neosynthesized inactive polypeptides into biologically active peptides. The first are generally lysosomal and the second are non-lysosomal proteases.

Ca^{2+} is widely accepted as a second messenger and regulator of many biochemical activities in the cell. Its action is mainly exerted through Ca^{2+}-binding proteins [1]. Ca^{2+}-binding proteins having proteolytic function, Ca^{2+}-dependent cysteine proteases or calpains have been described in the mammalian cells (for refs. see [2–6]). They are non-lysosomal neutral proteases having a cysteine protease domain homologous to proteases of the papain family and a Ca^{2+}-binding domain having amino acid sequence homology with calmodulin-like proteins [3]. These proteases are known to exert a wide variety of physiological functions in mammalian cells, such as enzymatic regulation, remodeling and disassembly of the cytoskeleton and cleavage of hormone receptors [2–6]. In mammalian cells there are two types of Ca^{2+}-dependent proteases: type I is active in the presence of micromolar calcium ions and type II requires millimolar calcium for the activation of enzyme activity [2–6]. Using specific antibodies the localization of both types has been studied in skeletal muscle cells [7–11], pig kidney cells [12], human pancreas and pituitary gland [13,14], rat cerebellum [15] and in proliferating cell lines [16–18].

In *Allomyces arbuscula*, a primitive eukaryote, the major proteolytic enzyme during the vegetative phase is a Ca^{2+}-dependent protease which seems to be the functional analogue of mammalian and avian calpains [19–21]. The enzyme is a 43–40 kDa peptide and requires millimolar calcium for its in vitro activity. Although cell fractionation experiments have shown that it is a cytosolic enzyme (unpublished results), its precise localization is not yet known. With highly specific antibodies raised in rabbit we have studied its localization in *A. arbuscula* and the results are presented in this report.

2. Materials and methods

2.1. Organism, cultural conditions and enzyme purification

A. arbuscula, the experimental organism was grown in GCY medium [22] and induced to differentiate zoospores according to procedure described earlier [19]. To obtain germinating cells, zoospores were inoculated in GCY medium and grown for 4–6 h with forced aeration. Enzyme was purified as described earlier [20].

The composition of the buffer used for enzyme purification was as follows: the MOPS buffer (pH 7.0) contained 20 mM MOPS, 2 mM EDTA, 2 mM EGTA, 3 mM MgCl, 1 mM DTT, 10 mM mercaptoethanol, 0.5 mM PMSF, 1 mM benzamidine, 5 μM leupeptine, 0.25 μg/ml pepstatine and 10 ml trasylol (215 μM). The elution buffer in the Ca^{2+} Phenyl-Sepharose column chromatography [23] was 20 mM Tris-HCl (pH 7.5) containing 10 mM EGTA and 0.1 M NaCl. Proteolytic activity of the enzyme in solution and in SDS-PAGE-gelatine was assayed as described earlier [20].

2.2. Polyclonal antibodies

Purified enzyme was electrophoresed on preparative 12.5% SDS-PAGE. The 43 kDa band was located by immersing the gel in 4 M Na-acetate according to Higgins and Dahmus [24], excised and homog-

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enized separately. The peptide was pure as judged by re-electrophoresis of a small amount of the preparation. The homogenized fraction was diluted with Freund's complete adjuvant and injected subcutaneously in rabbit. Second and third booster injections were made at three-week intervals. At the end of every third week a small amount of blood was taken and the titer of antibodies in the serum was quantitated by reacting the diluted serum to the purified enzyme by immunoblotting. The titer was already strong after the third week of the first injection. The antibodies from the serum were purified according to Dayton and Schollmeyer [7] with some modifications. The pooled fractions from the DEAE columns were mixed with the protein-A Sepharose and shaken for 3 h in the cold room. The suspension was packed in a column and the resin was washed with the binding buffer (20 mM Tris-NaCl, pH 8.0, containing 150 mM NaCl). Bound IgGs were eluted with 0.1 M Na-citrate (pH 3.0) in tubes containing adequate amount of 2 M Tris (pH 9.0) to immediately neutralize the eluate.

2.3. Purification of Ca^{2+}-dependent protease-specific antibodies

The procedure used was essentially as described by Beckerle et al. [25]. Briefly, the 43 kDa peptide was separated on 12.5% SDS-PAGE electrophoresis and transferred to nitrocellulose by electroblotting [26]. The band was excised and incubated first for 1 h in 3% gelatine in TBS (50 mM Tris-HCl, pH 7.5 and 0.5 M NaCl) at 25°C and then for 4 h at room temperature in purified antiserum diluted 1:4 in TBS. After this the excised band was washed twice in TBS and then the bound antibodies were eluted immediately with 2 M Tris base.

2.4. Electrophoresis, protein transfer and immunoblotting

SDS-PAGE electrophoresis was done according to Laemmli [27]. Acrylamide concentration in the stacking and separating gels was 5% and 12.5%, respectively. After electrophoresis the proteins were transferred overnight at 15 V/cm in a Bio-Rad electroblotting apparatus equipped with cooling device to nitrocellulose membrane (Schleicher and Schuell, GMBH, Dassel, Germany; pore size 0.45 μM), using the buffer system described by Burnette [26].

The transfer of proteins was checked by staining the filter with 0.05% (w/v) Ponceau red in 3% TCA. After a treatment with 5% BSA in TBS-Tween (0.5%), the filter was incubated in a dilute solution (usually 1:1000) of purified primary antibodies for at least 4 h at room temperature or overnight in the cold room (4°C), washed 3 times in TBS-Tween and re-incubated for 1 h at room temperature in a dilute solution (1:2000) of donkey anti-rabbit IgG conjugated to horseradisch peroxidase (Amersham). The filter was washed again and the peroxidase activity revealed with 0.025% 3-3'-diaminobenzidine tetrachloride (DAB) in 100 mM Tris-HCl (pH 7.5) containing 0.03% H_2O_2. The colour development was stopped by thorough rinsing with distilled water.

2.5. Fixation, wall digestion, membrane permeabilization

The cells were fixed with 4% *p*-formaldehyde in 10 mM Imidazol buffer (pH 7) containing 100 mM KCl, 4 mM $MgCl_2$, 4 mM EGTA and 1 mM ATP for 45 min at room temperature and then washed for 15 min in the same buffer. For partial digestion of the cell wall, the cells were incubated for 1 h at 40°C with 5 mg/ml of lysing enzyme (Sigma) in the same buffer. Digestion was stopped by rinsing for 10 min each in the above buffer and in TBS (10 mM Tris, 0.15 M NaCl, pH 7.5). The cell membrane of digested cells was permeabilized with 0.25% Triton X-100 in TBS buffer for 5 to 10 min at room temperature. The cells were finaly rinsed for 25 min in TBS buffer and used for immunofluorescence microscopy.

2.6. Immunofluorescence microscopy

Coverslips treated with 0.1% poly-L-lysine (MW 393,000; Sigma) in distilled water were loaded with 50 μl of the mycelial suspension. The cells were allowed to settle on the coverslip for 30 min, primary antibodies were added at 1:30 dilution in TBS and preparations were incubated overnight with anti-Ca^{2+}-dependent protease antibody at 4°C inside a Petri dish to avoid desiccation. Following a rinse with TBS for 25 min, the preparations were incubated with rhodamine conjugated (TRITC) to goat anti-rabbit immunoglobulin (Nordic Biogenzia, Lemania, Lausanne, Switzerland) at a dilution of 1:40 in TBS. A Leitz Orthoplan Microscope, Ernst Leitz, Wetzlar (Germany) equipped with fluotar optics and selective filter combination was used for viewing the TRITC-rhodamine patterns. Photographs were taken on HP5 Ilford black and white 400 ASA film.

3. Results and discussion

Purified Ca^{2+}-dependent protease showed two bands in SDS-PAGE, respectively of M_r 43 and 40 kDa (Fig. 1A). Affinity-purified 43 kDa antibody reacted to both peptide bands indicating that they share common epitopes (Fig. 1B). This confirmed our earlier conclusion that the 40 kDa peptide was derived from the 43 kDa protein [28].

Antibodies, either in the serum or in the affinity-purified form, were also tested for specificity in immunoblotting reactions with crude extract of actively growing mycelia. As shown in Fig. 1C, a strong band corresponding to M_r of 45 kDa and two very faint bands of 43 and 40 kDa were recognized indicating that the enzyme in the native state has a molecular weight higher than the 43–40 kDa obtained in SDS-PAGE of purified protein. This proteolytic modification of the native enzyme during purification could not be avoided even by use of higher concentrations of inhibitors in the buffer solutions.

Having ascertained the specificity of the antibodies to the Ca^{2+}-dependent protease in crude extracts, we looked for the location of the enzyme in vegetative mycelia by immunofluorescence. As a negative control for the fluorescence background we used the preimmune serum. The results indicated that the enzyme was localized in the cytosol and along the cytoplasmic membrane (Fig. 2d). Its presence in the cytosol seemed to be higher in the tip region of the hyphae. The fluorescence observed in the control (preimmune serum) was insignificant (Fig. 2b), showing the specificity of the antigen–antibody reaction. As a control of our methodology we studied the localization of actin which is known to be predominantly apical

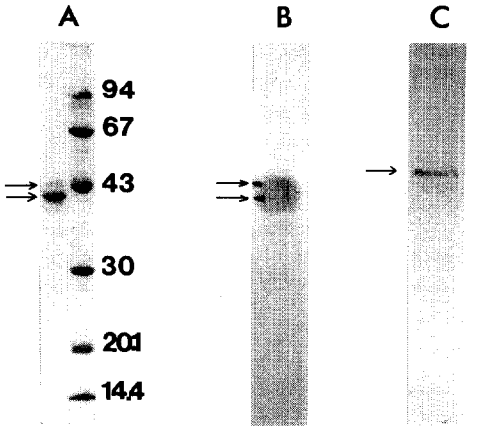

Fig. 1. Immunoblotting of purified enzyme and crude extract of *A. arbuscula*. (A) Coomassie blue staining of 12.5% SDS-PAGE of purified Ca^{2+}-dependent protease. (B) Immunoblotting of purified protease showing 43 and 40 kDa bands. (C) Immunoblotting of crude extract of *Allomyces* showing a prominent 45 kDa and faint 43–40 kDa bands.

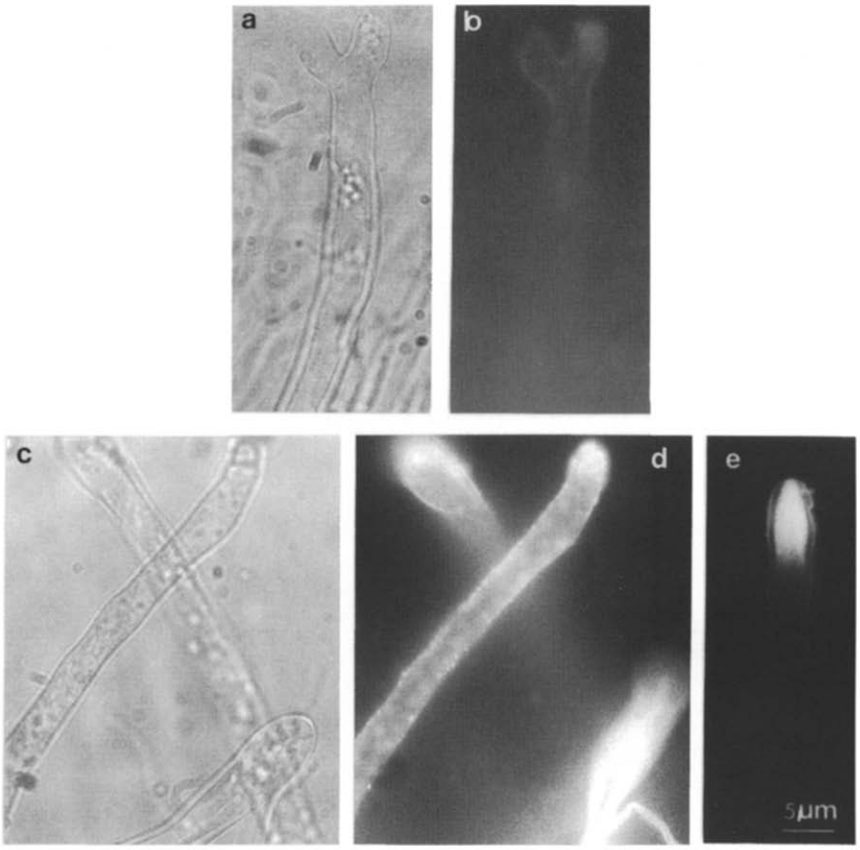

Fig. 2. Immunolocalization of Ca^{2+}-dependent protease in the exponentially growing mycelia. Mycelia treated with preimmune serum observed under bright field illumination (a) and UV light (b), showing respectively the state of the hyphae and the fluorescence background. Mycelia treated with specific Ca^{2+}-dependent protease antibody observed under bright field illumination (c), and in UV light (d) for specific immunofluorescence. Note the accumulation of the protease antigen at the hyphal tips and along the cellular membrane. Control immunofluorescence of actin (e) shows its apical distribution.

[29,30]. As expected, actin appeared in the form of a cap in the growing apex (Fig. 2e). The light microscopy of the same hyphae is shown in Figs. 2a and c, indicating that the state of mycelia was not deformed during preparation.

In a previous report we have shown that the enzyme activity is developmentally regulated, i.e. it is present in the vegetative phase but disappears when the mycelia are induced to develop zoosporangia and zoospores [19]. We therefore studied the cell-free enzyme activity (partially purified by the DEAE ion-exchange column), the presence of antigen and in situ localization of the enzyme starting from zoospores to germinating zoospores and exponentially growing mycelia. As expected, zoospores had no Ca^{2+}-dependent proteolytic activity which started to appear during germination and achieved its maximum expression during exponential growth (Table 1). These results corroborated those obtained by immunoblotting of the crude extracts (Fig. 3) indicating that the enzyme activity was related to the presence of the antigen and not to any specific protease inhibitor. The confirmation of these results was also obtained by immunofluorescence experiments which showed a clear absence of this antigen in the zoospores (Fig. 4 a–c) and its presence in germinating spores which was polarized in two growing zones: rhizoids and germ tube formation sites (Fig. 4f and h, see arrows).

Table 1
Evolution of Ca^{2+}-dependent protease in cell types of *A. arbuscula*

Cell types	Ca^{2+}-dependent specific enzyme activity (units/mg)*	Presence of Ca^{2+}-dependent enzyme antigene
Zoospores	0	–
Germinating spores (6 h culture)	75	+
Growing hyphae (18 h culture)	178	+++

* The figures shown represent a mean-value of 4 independent measurements.

In mammalian cells, two types of Ca^{2+}-dependent proteases (calpains) have been described, type I and type II, requiring respectively μM Ca^{2+} and mM Ca^{2+} for enzyme activation. Intracellular distribution of these enzymes was studied by Murachi [4] by immunofluorescence and immunogold staining. A recent study in mitotic cells [18]

suggested that both types are associated with microtubules of spindles. There is evidence for in vitro and in vivo proteolysis of MAPs (microtubule-associated proteins) by these enzymes [29,30], suggesting a functional correlation of this colocalization. Further, in myoblasts cells, type I has been shown to be localized in the cytoplasm but type II appeared to be predominantly associated with the plasma membranes in the fusion competent cells [10]. This peripheral distribution in fusion-competent compared to non-fusing variants, was suggested to have a role in fusing events of differentiating myoblasts.

The resolution of cytosolic distribution of mM Ca^{2+}-dependent protease in our study is not sufficient to associate it to microtubules although we have shown that in vitro tubulin is specifically and rapidly degraded by the enzyme (M. Ojha, unpublished results). However, the significance of apical localization of the enzyme can be understood through the nature of hyphal growth which is known to require the plasticity of cytoskeletal elements in this region.

The predominantly plasma membrane and cap like cytosolic localization of Ca^{2+}-dependent protease in *Allomyces* in the hyphal tip regions indicate that this zone of hyphal elongation is rich in the enzyme and that it might somehow be related to the hyphal extension, presumably by acting on cytoskeletal proteins at the apex. It is also known that there is an apico-basal Ca^{2+} gradient in the apical region of the hyphae with tips being richer in Ca^{2+} ions. Further, it has been suggested that Ca^{2+} acts as a signal through waves of transient increase in cytosolic free Ca^{2+} concentration at the tips [31]. Thus,

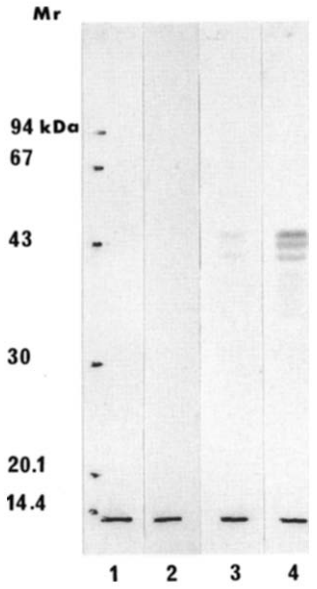

Fig. 3. Immunoblotting of Ca^{2+}-dependent protease from crude extracts of *A. arbuscula* prepared from different stages of culture development. Molecular weight markers (1); proteins from zoospores (2); germinating zoospores (3) and exponentially growing mycelia (4), showing respectively the absence, faint presence and strong presence of the antigen in the zoospores, germinating zoospores and exponentially growing mycellia.

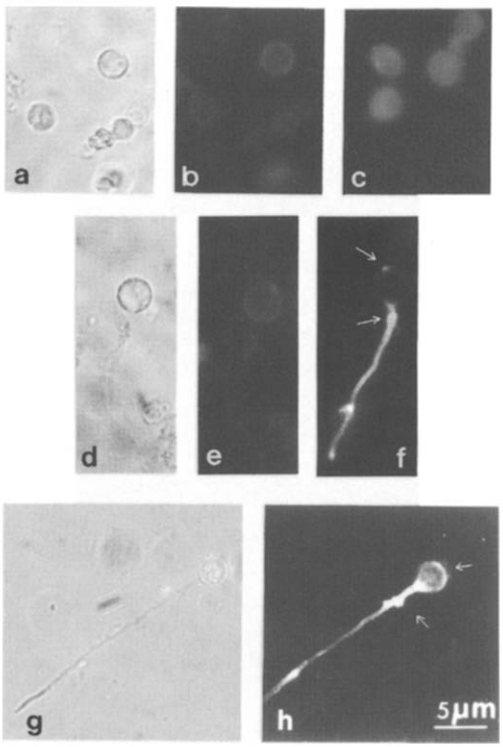

Fig. 4. Immunocytochemical localization of the Ca^{2+}-dependent protease in *A. arbuscula*. Zoospores treated with preimmune serum under: bright field illumination (a); UV light (b) and treated with specific serum (c), showing almost no fluorescence. Germinating zoospores treated with preimmune serum under: bright field illumination (d); UV light (e), showing the low fluorescence background. Germinating zoospores treated with specific antiserum under: bright field illumination (g), and UV light (f,h). Arrows indicate the polarized appearence of protease in elongation zones.

it is possible that this transient increase in Ca^{2+} in the apical region, where there is a high concentration of the enzyme, activates or deactivates the protease which has an absolute requirement of Ca^{2+} for its activity. The enzyme in turn would act on cytosolic proteins, as well as membrane-associated proteins, and permit the hyphal elongation.

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