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Isolation and electrophoretic analysis of surface proteins of the zygomycete *Absidia glauca*

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Methods for isolation and electrophoretic analysis of surface proteins from the mucoraceous fungus *Absidia glauca* are described. By analysing the set of cell wall associated proteins which can be extracted with 1 M LiCl, we could show marked differences between submersed and aerial mycelium. Furthermore, during differentiation of the aerial mycelium protein patterns change remarkably. We were able to demonstrate the existence of mating type specific proteins, the most prominent of which is a 15 kDa polypeptide expressed in submersed as well as in aerial hyphae of the (+) mating type.

Cell wall; Surface protein; Glycoprotein; Sexual development; Zygomycete; (*Absidia glauca*)

1. INTRODUCTION

Absidia glauca is a typical representative of mucoraceous fungi of the class zygomycetes. Besides their biotechnical relevance, zygomycetes have proved to be valuable model systems for a number of fundamental biological questions. Well-known examples are *Phycomyces blakesleeanus*, the light-dependent growth regulation of which has mainly been studied [1], and *Mucor mucedo*, a useful model system for investigating the genetics of sexual spore formation [2] and the physiology of mating type specific hormones [3].

We are mainly interested in the developmental pathway towards the formation of sexual zygospores in the heterothallic model organism *A. glauca*. Compared with the more established systems *P. blakesleeanus* and several *Mucor* species, this fungus offers several experimental advantages [4]. Furthermore, efficient experimental protocols for protoplast fusion and transformation have recently been described [4,5]. Sexual interaction of zygophores from complementary mating types of mucoraceous fungi seems to be controlled generally by at least two different hormone systems. Derivatives of trisporic acid induce the formation of early sexual structures [6], whereas a system composed of volatile zygotropic substances probably mediates the directed growth of complementary zygophores towards each other [7]. Bu'Lock [8] proposed that in addition to hormonal interactions, specific surface components might be involved in the fusion process of gametangia. A detailed chemical analysis of the cell wall composition of *A. coerulea* provided evidence that proteins constitute approx. 5% of the cell wall material [9]. Immunological studies with antibodies against total hyphae and against a fraction enriched in zygophores from *M. mucedo* did indeed reveal zygophore specific surface material [10]. However, no evidence for mating type specific surface determinants could be found by this immunological approach. We decided to isolate surface specific proteins from both mating types of *A. glauca* grown separately, as well as from mating cultures and to look for mating type specific determinants by means of different electrophoretic techniques.

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2. MATERIALS AND METHODS

2.1. *Strains and growth conditions*

Both mating types of *A. glauca* (CBS 100.48(+) and CBS 101.48(−)) were obtained from the Centraalbureau voor Schimmelcultures in Baarn (The Netherlands). Liquid cultures for the production of submersed mycelium were inoculated with 2×10^4 vegetative spores per ml and grown in complete medium as previously described [11] for approx. 44 h. Aerial mycelia were grown on complete medium in petri dishes on the surface of filter paper disks for 3 to 23 days at room temperature (inoculum: 5×10^6 spores per petri dish).

2.2. *Extraction of surface proteins*

Mycelia from liquid cultures were washed once with distilled water on a Büchner funnel and extracted at room temperature for 15 min on a rotary shaker with a solution containing 1 M LiCl, 20 mM sodium phosphate, pH 7.0, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). For processing of aerial mycelia the washing step was omitted. This protocol is a modification of a procedure for the isolation of cell wall glycoproteins from *Chlamydomonas reinhardtii* [12]. For further analysis proteins were either precipitated with trichloroacetic acid or concentrated by ultrafiltration on Amicon YM10 membranes and desalted by dialysis against destilled water. Protein concentrations were determined according to Bradford [13].

2.3. *Preparation of antibodies*

Antibodies against surface proteins from both mating types and against a preparation from mated cultures were raised in rabbits. Typically, 0.5 mg protein in complete Freund's adjuvants were injected intracutaneously into several portions followed by an intravenal booster injection of the same amount four weeks later. Unfractionated sera were employed for immunoblot analysis [14].

2.4. *Electrophoretic techniques*

Proteins were either analysed on one-dimensional, 17.5% acrylamide gels according to Laemmli [15] or in two dimensions following the procedures of O'Farrell [16]. For isoelectric focusing, ampholytes in the pH range 3–10 (Pharmacia) were used according to the manufacturer's instructions. Nondenaturing 17.5% gels were run in 25 mM Tris-HCl at pH 6.8. Proteins were either made visible by silver-staining [17] or by staining of immunoblots with protein A coupled peroxidase (BioRad). Glycoprotein staining was performed on nitrocellulose affinoblots by successive binding of concanavalin A and horseradish peroxidase [18]. All electrophoresis experiments were performed with protein preparations from seven independently grown *Absidia* cultures.

3. RESULTS

3.1. *Comparison between submersed and aerial mycelium*

An important differentiation step in the growth cycle of any filamentous fungus is the development of aerial hyphae starting from substrate mycelium which from an experimental point of view can essentially be regarded as equivalent to mycelia grown submersed in liquid culture. One of the main physiological tasks for substrate hyphae is the uptake of water and nutrients, whereas the aerial mycelium usually forms structures for reproduction and propagation. That both types of mycelia from *A. glauca* differ from each other biochemically can be anticipated: aerial mycelia exhibit a much higher degree of hydrophobicity, which is perhaps reflected by the higher content of lipids in sporangiophores compared to substrate mycelium [19].

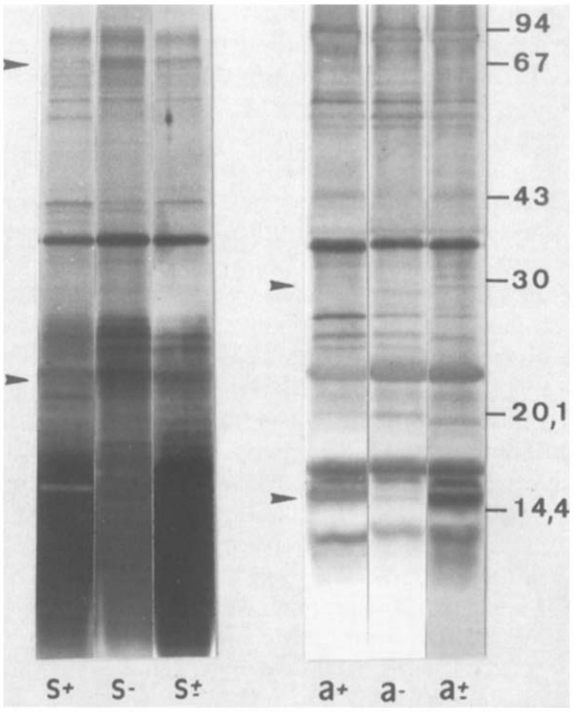

Fig.1. Comparison of surface proteins from aerial (a) and submersed (s) mycelia of *A. glauca*. Approx. 5 μg of each protein preparation were analysed on 17.5% SDS-acrylamide gels. Marker proteins were from Pharmacia and are indicated by bars. Mating type specific proteins (15, 22, 66 kDa) and a protein specifically expressed during gametangia formation (29 kDa) are marked by arrowheads.

Surface protein preparations from submersed and aerial mycelia were analysed by silver staining of polyacrylamide gels (fig.1). We could show that our protein preparations obtained by washing total hyphae with 1 M LiCl are essentially free from intracellular contaminants by two lines of evidence.

No prominent bands typical of intracellular proteins could ever be detected in our preparations and, on the other hand, proteins from lysed protoplasts showed very little cross-reaction with antibodies raised against LiCl-extracted material. The only weak interactions were observed with a few high molecular mass bands which did not comigrate with any of the authentic surface proteins (not shown).

Proteins from submersed and aerial mycelium respectively show strong similarities, particularly a prominent band with a molecular mass of 34 kDa seems to occur in very similar concentrations during all growth stages. Nevertheless many marked differences can be pointed out. Both types of mycelia exhibit several stage specific proteins. Overall, preparations from liquid cultures contain considerably more different polypeptides, especially in the low molecular mass range below 25 kDa. All major surface proteins with molecular masses above 22 kDa from both growth stages proved to be glycoproteins as shown by their capability to bind concanavalin A in affinity blotting experiments. Only a few bands in the lower molecular mass range could be stained by this procedure. Antibodies raised against surface proteins from aerial mycelium usually cross-react with proteins from submersed cultures. This does not necessarily mean that all of these proteins are homologous. By chemical deglycosylation of surface proteins with hydrofluoric acid [20] followed by Western blot analysis, we could demonstrate that most of the antibodies are directed against sugar moieties of glycoproteins. In accordance with this observation, polypeptides in the lower molecular mass range gave rise only to very weak signals on immunoblots.

3.2. *Growth stage specific surface proteins*

The patterns of surface proteins from aerial mycelia are not constant during development. Fig.2 shows a time course of surface protein expression for the (+) mating type between the first appearance of aerial hyphae and the completion of maturated vegetative sporangia. Four bands with molecular masses of 21, 48, 56 and 65 kDa disappear with increasing age, whereas eight proteins are formed later during development. The relative amounts of four of these proteins (21.5, 24, 60 and 68 kDa) increase until the 11th day of growth;

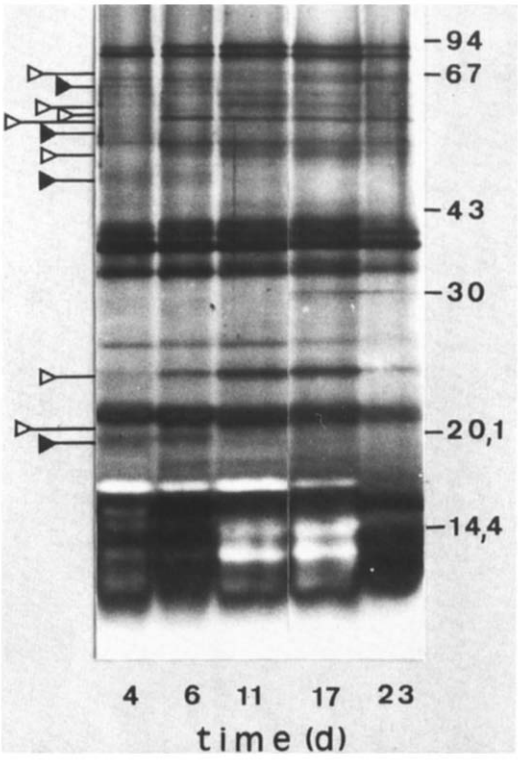

Fig.2. Growth stage specific surface proteins from aerial mycelia. Surface proteins were prepared from the (+) mating type after growth on solid medium for the time indicated. Same amounts of proteins (5 μg) from each preparation were analysed as in fig.1. Bands whose relative amounts decrease with culture time are marked by open triangles; increasing proteins are labeled by filled triangles.

other proteins (52, 58 and 59 kDa) reach their highest concentration around the 6th day of development, which coincides roughly with the completion of sporangia formation.

3.3. *Mating type specific proteins*

It may be assumed that opposite mating types of mucoraceous fungi exhibit some differences in the molecular architecture of their hyphal surface. These differences might either reflect the cooperative enzymatic complement of both mating types for the production of sexual hormones of the trisporic acid system [3], or they might be a consequence of complementary surface proteins used for intersexual contact formation during gametangial fusion.

A comparison of surface proteins from submersed mycelia on SDS gels reveals that two bands with

molecular masses of 22 and 66 kDa, respectively, light up stronger in preparations from the (−) mating type (marked by arrowheads in fig.1). A better resolution is possible if proteins are separated in two dimensions and stained on nitrocellulose blots using antibodies against total surface proteins as shown in fig.3. The most obvious difference however is caused by a (+) type specific 15 kDa protein which is completely lacking in preparations from the (−) type. No additionally induced proteins could be found when both mating types were simultaneously grown in the same culture flask.

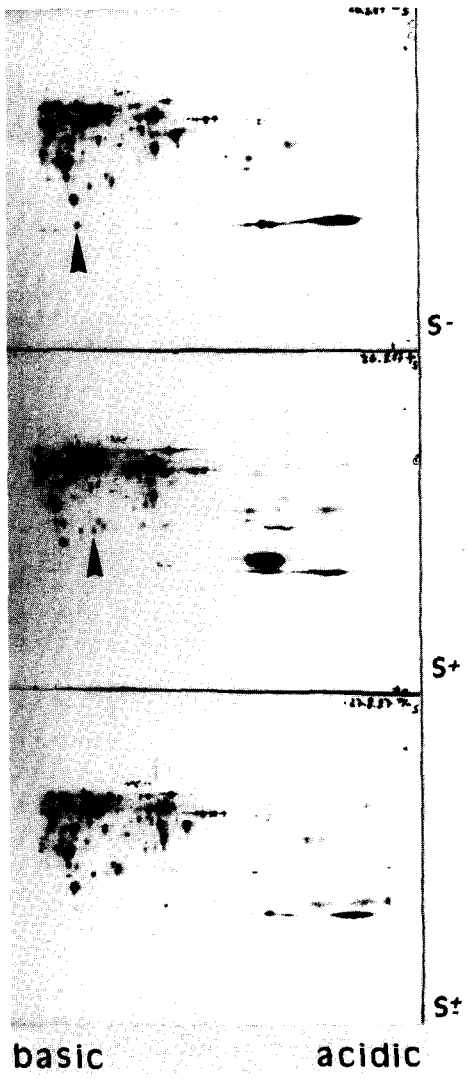

Fig.3. Two dimensional gel electrophoresis of surface proteins from submersed mycelium. Top: (−) type; middle: (+) type; bottom: cocultivation of both mating types. Separation in the first dimension was by isoelectric focusing in the pH range 3–10; in the second dimension proteins were run on SDS gels. Staining was performed on nitrocellulose blots by consecutive binding of antibodies against surface proteins from mated cultures and protein A-coupled horseradish peroxidase; 4-chloro-1-naphthol was used as substrate. Proteins in the range between 22 and 100 kDa are stained by this procedure. Mating type specific polypeptides are marked by arrowheads.

The analysis of proteins from aerial mycelia revealed one rather prominent mating type specific protein with a molecular mass of 15 kDa. This protein can clearly be resolved if the analysis is performed on native polyacrylamide gels at pH 6.8. Under these conditions very few polypeptides including the 15 kDa (+) type specific protein migrate towards the cathode. This protein (marked by an arrowhead in fig.4A) comigrates with the 15 kDa protein specific for (+) type mycelia grown in liquid culture. Although some protein isolated from the (−) mating type runs to the same position in these gels, we could show that this material is not identical with the (+) specific 15 kDa protein. By cutting lanes from native gels and subsequent electrophoresis of the proteins into an SDS gel we could resolve the material from the (−) mating type into several smaller polypeptides whereas the (+) specific band proved to consist of a single protein chain with a molecular mass of 15 kDa. This protein does not contain sugar moieties detectable by binding of concanavalin A and it does not react with antibodies against total surface proteins from aerial mycelia of any mating type.

In addition to this mating type specificity we could demonstrate the appearance of a faint band with a molecular mass of 29 kDa in protein preparations from mated cultures.

4. DISCUSSION

Cellular recognition and sexual agglutination in the ascomycete yeasts *Hansenula wingei* [21] and *Saccharomyces cerevisiae* [22,23] are at least to a certain extent mediated by specific glycoproteins at the cellular surface. The involvement of surface proteins in differentiation processes on a completely different evolutionary stage has convincingly been shown in the cellular slime mold *Dictyostelium discoideum* [24].

Unfortunately, essentially no information is

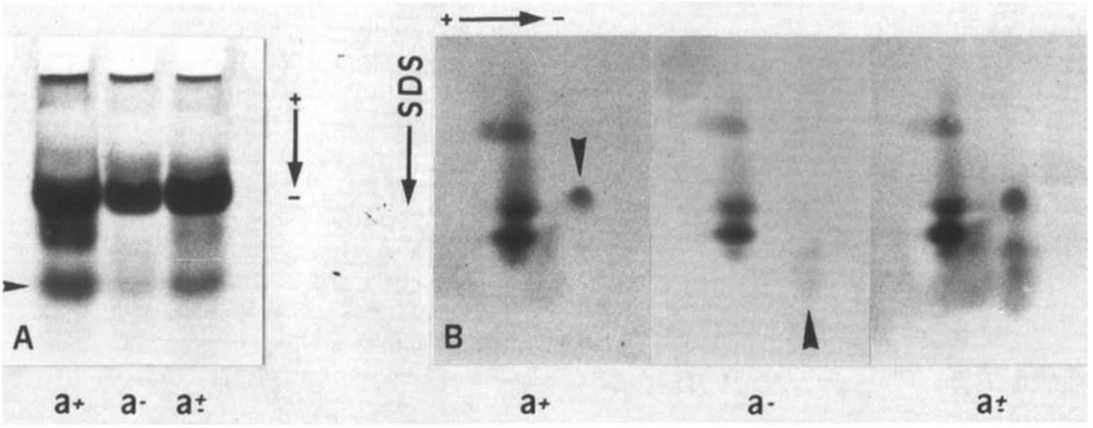

Fig.4. Identification of a (+) type specific 15 kDa protein. (A) Separation of surface proteins from aerial mycelia at pH 6.8 under nondenaturing conditions. (B) Lanes from native gels were cut with a razor blade, equilibrated with SDS-containing electrophoresis buffer and proteins were run into a SDS-containing gel. The material was made visible by silver staining.

available on the relevance of macromolecules situated on the hyphal surface for differentiation processes of filamentous fungi. Although the involvement of surface determinants in cellular recognition processes between sexually compatible strains has been proposed by mycologists quite a few years ago [8], we still have very little information on the biochemistry and biological specificity of such molecules. One of the main problems in investigating the role of surface proteins in differentiation programmes and intercellular communication is the almost complete lack of methods for their isolation and analysis. Among the few approaches for gaining information about the molecular architecture of hyphal surfaces are immunological studies on cell wall associated polysaccharides [25] and ultrastructural investigations by scanning electron microscopy [26]. In this report we propose a reliable, easy procedure, based on washing intact fungal mycelia with 1 M LiCl, allowing the isolation of surface proteins without any detectable contaminations with intracellular material (see section 3.1). The method has been optimized with regard to salt concentration, extraction time and temperature (not shown) and has proved its value with respect to the analysis of more than 20 different fungi from several families of zygomycetes. It turned out to be possible to differentiate all of the tested strains solely on the basis of their specific surface protein patterns (in preparation). Presently, we cannot distinguish between proteins loosely bound to the mycelial surface and material that might represent tightly bound integral cell wall components. In principle, it might even be possible that the extraction procedure liberates some proteins from the hyphal membrane. We could however demonstrate that cell walls from *A. glauca* contain some protein material which cannot be extracted by 1 M LiCl. Fractions enriched for cell walls were prepared by mechanical disruption of LiCl-washed mycelia from liquid cultures followed by extensive extraction with phosphate buffered saline in order to remove most of the cytoplasmic material. SDS extracts of this material gave rise to a somewhat diffuse pattern on polyacrylamide gels with only a few prominent bands stemming from cytoplasmic contaminants. No proteins with electrophoretic mobilities typical for LiCl-extracted material could be found. However, antibody staining revealed distinct bands of high molecular mass which are clearly different from the normal LiCl pattern.

Another point that should be discussed for critical evaluation of the extraction procedure is the potential generation of artifacts by extracellular proteases that might cleave surface proteins during preparation. We could show by several lines of evidence that protease activities in submersed *Absidia* cultures are very low and cannot be responsible for degradation of proteins during the isolation procedure. No protease activity could be measured in culture filtrates or surface

protein preparations from submersed mycelia with Blue Hide powder azure as substrate [27]. Additionally, no degradation of bovine serum albumin could electrophoretically be detected in such preparations even after incubation at room temperature for 14 h. Identical results were obtained in parallel assays with protein preparations dialysed against water, and we could find no differences between assays with or without PMSF. A set of perhaps more critical degradation tests has been performed with LiCl-extracted proteins. Incubation of surface proteins for 14 h in the absence of PMSF followed by electrophoretic analysis gave no evidence for proteolytic degradation. Even the addition of crude culture filtrates did not considerably change protein patterns; only one band with a molecular mass of 40 kDa disappears after incubation at room temperature for several hours. Even if we incubate surface proteins with complete *Absidia* mycelia the protein pattern remains constant for more than 2 h with the exception of the 40 kDa band mentioned before. After this time the normal pattern of surface proteins is increasingly superimposed by an additional set of proteins which probably represents exoproteins secreted into the medium by *A. glauca*.

By comparing preparations of surface proteins from both mating types of *A. glauca* we provided evidence for the occurrence of mating type specific proteins. The most prominent difference between mating types is a (+) type specific protein with a molecular mass of 15 kDa, which can be found in aerial hyphae as well as in mycelium grown in liquid culture. Presently, we have no experimental data on the functional significance of this protein. However, it is highly improbable that it is involved in the process of cellular recognition between mating types, because no evidence for a topologically defined expression could be gathered. On the other hand, it can by no means be excluded that the 15 kDa protein represents one of the enzymes involved in the biochemical pathway towards trisporic acid and thus would nevertheless directly be involved in the process of sexual development. The synthesis of trisporic acid derivatives is accomplished by the complementary action of enzymes from both mating types [3] and is believed to take place primarily in the substrate hyphae of mucoraceous fungi.

With our electrophoretic analysis of cell wall associated proteins, we cannot support the idea that considerable amounts of new proteins are incorporated into the hyphal cell wall during formation of gametangia, which might be inferred from an immunological study with *M. mucedo* [10]. It is however possible that differences between vegetative and reproductive structures found by immunological in situ techniques do not necessarily reflect the synthesis of new proteins. Differential modification of the same protein skeleton could easily be responsible for changes in function and different immunological properties.

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