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THE DERMATOPHYTE *TRICHOPHYTON RUBRUM* SECRETES AN EDTA-SENSITIVE ALKALINE PHOSPHATASE ON HIGH-PHOSPHATE MEDIUM

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ABSTRACT

In this communication, we show that the growth of isolate H6 of the dermatophyte *Trichophyton rubrum* on non-buffered medium and under saturating phosphate conditions is dependent on the initial growth pH, with an apparent optimum at pH 4.0. In addition, irrespective of the initial growth pH, the pH of the medium altered during cultivation reaching values that ranged from 8.3 to 8.9. Furthermore, this isolate synthesized and secreted almost the same levels of an alkaline phosphatase with an apparent optimum pH ranging from 9.0 to 10.0 when grown on both low- and high-phosphate medium. Also, this alkaline phosphatase is activated by Mg^{2+} and is EDTA-sensitive. On the other hand, the very low levels of the enzyme retained by the mycelium grown on buffered medium at pH 5.0-5.2 suggest that this enzyme is encoded by an alkaline gene, i.e., a gene responsive to ambient pH signaling.

Key words: alkaline phosphatase, ambient pH, dermatophytes, enzyme secretion, *Trichophyton rubrum*, Pi sensing

INTRODUCTION

Trichophyton rubrum, a filamentous fungus which causes infections in human skin and nails, is recognized as cosmopolitan and is one of the most frequently encountered dermatophytes (1,4). However, this fungus which normally causes well-characterized superficial infections has been recently described as an invasive pathogen in immunocompromised hosts (12).

Although several factors contribute to the pathogenicity of dermatophytes, the successful initiation of infection is a process closely related to the capacity of the infecting fungus to overcome the host resistance mechanisms, which include the fungistatic fatty acids and the intact keratinized layers of the skin, and other physiochemical effectors such as moisture and skin pH (21). The capacity of dermatophytes to adhere to cell surfaces has been attributed to the presence of glycoproteins containing mannans in the cell wall of these microorganisms, and the greater their adherence capacity, the greater their invasive ability and virulence (26). However, for dermatophytes to be successful in their installation in the host the arthroconidia must germinate very rapidly and the hyphae penetrate the body surface or they will be lost by continuous desquamation of the epithelium.

Once established, the dermatophytes must scavenge nutrients for growth, an adaptive response to the environment relatively well known in model fungi such as *Neurospora crassa* and *Aspergillus nidulans*. For example, the use of nucleic acids as sources of inorganic phosphate (Pi) is based on the induction of structural proteins, permeases, and enzymes of the cell wall, in addition to the secretion of a variety of proteins and hydrolytic enzymes such as nucleases and acid and alkaline phosphatases in response to both Pi starvation and environmental pH signaling. This metabolic machinery permits the fungi, and other microorganisms, to use these macromolecules as sources of essential nutrients within a broad ambient pH range (8,11,17,19). Thus, it is also important to understand the metabolic responses that govern homeostatic pH and extracellular pH sensing in dermatophytes, since the pathogenicity of many microorganisms has been demonstrated to depend on the sensing of ambient

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pH in the host and on the ability to adapt, which involves installation, development, and survival in the host.

In order to investigate further the response of dermatophytes to ambient pH, our first aim was to measure the derepression and secretion of alkaline phosphatases (APases) synthesized by isolate H6 of the dermatophyte *T. rubrum* on the basis of enzymatic activity. We observed the secretion of apparently only one APase irrespective of the extracellular Pi levels, which is possibly encoded by an alkaline gene, i.e., a gene responsive to ambient pH signaling.

MATERIALS AND METHODS

The clinical *T. rubrum* isolate (H6) obtained from a patient admitted to the University Hospital of the Faculty of Medicine of Ribeirão Preto, SP, Brazil, and identified by standard methods (16) was stored at room temperature in Sabouraud glucose agar medium (3) covered with sterilized water until the time of use.

After incubation of isolate H6 on Sabouraud glucose agar for 12 days at 28ºC, conidia and mycelium from three Petri dishes (14 cm diameter) were carefully harvested in 300 mL Sabouraud glucose liquid medium and cultured in an orbital shaker (160 rpm) at 28ºC. After shaking for 96 h the culture was centrifuged (15 min at 11,000 x g at 4ºC), the pellet resuspended in 50 mL 0.9% (w/v) NaCl and centrifuged again for 10 min at 11,000 x g at 4ºC. This procedure was repeated twice and the pellet free of Sabouraud medium was suspended in 50 mM 0.9% (w/v) NaCl. Twenty mL of this suspension was inoculated into 100 mM of low-(200 µM KH_2PO_4) and high-Pi (10 mM) minimal liquid medium of Cove (9). Low-Pi medium was prepared by adding 200 µM KH_2PO_4 and 10 mM KCl to Pi-free minimal medium (final concentrations). Both high- and low-Pi media were supplemented (final concentrations) with 50 mM glycine, 1% (v/v) glycerol, and 70 mM $NaNO_3$, and the pH was adjusted as desired. After incubation for 72 h in an orbital shaker (160 rpm) at 28ºC, the mycelium was harvested by filtration, washed with distilled water and pressed to remove excess liquid. It was then ground in a pre-cooled mortar with washed sand and 20 mM Tris HCl buffer, pH 8.9 [10 mL buffer (g mycelium)$^{-1}$], containing 1 mM PMSF and 1 mM benzamidine, 4ºC. The mycelial extract was centrifuged at 20,000 x g for 20 min, and the supernatant used for enzyme assays without further treatment (mycelial extract). An appropriate volume of the harvested culture medium was concentrated about 40 times by ultrafiltration through AMICON (YM10) membranes and used for enzyme assays without further treatment (culture medium).

The enzymatic activity was determined in 0.3 M Tris HCl, pH 8.9, containing one mM $MgSO_4$, using six mM ρ-nitrophenyl phosphate (PNP-P) as substrate at 37ºC, a cocktail used to assay the constitutive APase from *N. crassa* (14). One Unit of enzyme activity was defined a one ηmol substrate hydrolyzed min^{-1}. Specific activities were expressed as Units (mg dry weight mycelium)$^{-1}$. The S.D. was calculated from two replicates. At least two independent experiments were carried out. The buffers used to cover the pH range required were 0.1 M MOPS, pH 7.0-7.5, 0.1 M Tris HCl, pH 7.5-8.5, 0.1 M TAPS, pH 8.5-9.5, and 0.1 M CAPS, pH 9.5-10.5. APase activity was determined using the standard procedure.

RESULTS

Growth of isolate H6 of *T. rubrum* on a mixture of glycine and glycerol under saturating Pi conditions is dependent on the initial growth pH, with an apparent optimum at pH 4.0. In addition, irrespective of the growth pH, the pH of the medium altered during cultivation, reaching values that ranged from 8.3 to 8.9 (Table 1).

Table 2 shows that isolate H6 of *T. rubrum* synthesized and secreted almost the same levels of an APase when grown on both low- and high-Pi medium, as measured by the enzymatic

Table 1. Growth of isolate H6 of *Trichophyton rubrum* in non-buffered high-phosphate minimal liquid medium. The dermatophyte was cultured for 4 days in an orbital shaker (160 rpm) at 28ºC. For details see Materials and Methods.

Initial pH	Final pH	mycelial dry weight (g/100 mL medium)
3.0	8.3	1.8 ± 0.3
4.0	8.4	2.0 ± 0.3
6.8	8.5	1.4 ± 0.2
8.0	8.6	0.5 ± 0.1
9.0	8.9	0.2 ± 0.1

Table 2. Effect of culture conditions on the production and secretion of alkaline phosphatase in *Trichophyton rubrum*[a].

Culture conditions	mycelial dry weight (g/100mL)	Initial pH	Final pH	Alkaline Phosphatase[b]	
				Mycelial extract	Culture medium
High-Pi (10 mM)[c]	1.3 ± 0.2	6.8	8.5	70 ± 4	72 ± 1
Low-Pi (200 µM)[c]	0.8 ± 0.1	6.8	8.6	84 ± 3	93 ± 2
	0.4 ± 0.1	5.0	5.2	0.1 ± 0	0.1 ± 0
High-Pi (10 mM)[d]	0.6 ± 0.1	6.8	7.0	46 ± 2	14 ± 1
	0.8 ± 0.2	8.0	7.9	47 ± 3	13 ± 1

[a] Isolate H6 of the dermatophyte was grown in Sabouraud dextrose liquid medium under saturating phosphate conditions, transferred to low- and high-Pi minimal liquid medium, and then incubated for 3 days in an orbital shaker (160 rpm) at 28ºC; [b] Enzyme activity over PNP-P. Units (mg dry weight mycelium)$^{-1}$; [c] Non-buffered medium; [d] Buffered medium with 50 mM Na citrate (pH 5.0), 50 mM PIPES (pH 6.8), and 50 mM Tris-HCl (pH 8.0); For details see Materials and Methods.

hydrolysis of PNP-P. When the dermatophyte was grown on buffered medium the production and secretion of the enzyme was repressed at pH 5.0, and reduced at both pH 6.8 and 8.0 (Table 2). As previously observed for APases synthesized by other fungi, the pH activity profile showed an apparent optimum pH ranging from 9.0 to 10.0 for both mycelial and secreted enzymes, irrespective of the extracellular Pi levels (Fig. 1). Also, this APase is activated by Mg^{2+} and is EDTA-sensitive (Fig. 2).

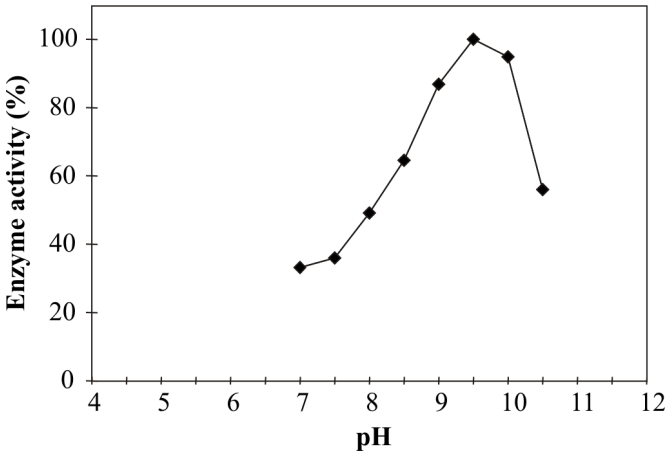

Figure 1. pH activity profile of alkaline phosphatase secreted by isolate H6 of *Trichophyton rubrum* grown in non-buffered high-phosphate medium at 28ºC, pH 6.8. For details see Material and Methods.

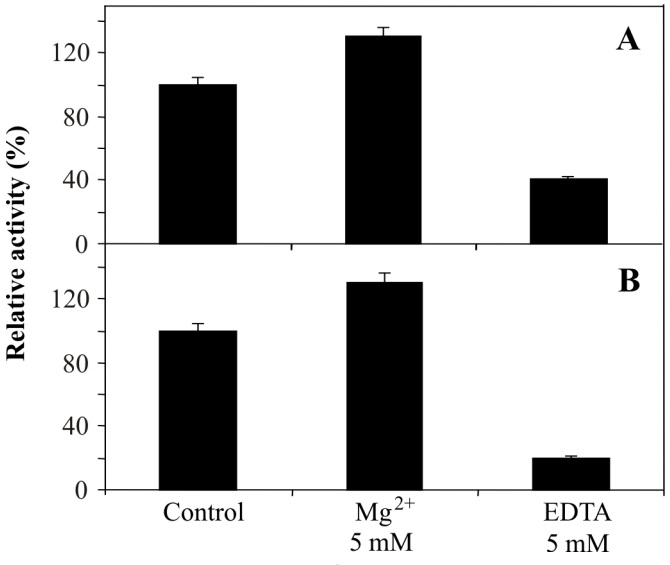

Figure 2. Effect of Mg^{2+} and EDTA on the *p*-nitrophenylphosphatase activity, pH 8.9, of alkaline phosphatase synthesized by isolate H6 of *Trichophyton rubrum* grown in high-phosphate medium at 28ºC, pH 6.8. **(A)** mycelium extract; **(B)** culture medium. For details see Materials and Methods.

DISCUSSION

The fungi, and probably all living organisms, synthesize a number of phosphatases which are necessary to scavenge Pi from medium containing nucleic acids as the sole phosphorus source, i.e., these enzymes are secreted in response to signals of the absence of Pi (22). The regulation of this adaptive response has proved to be highly complex, not only because of the identification of a large number of genes involved in the signaling of Pi starvation, but also because the synthesis and secretion of these enzymes are under the action of nitrogen, carbon and pH regulatory circuits (8,13,19). The pH regulatory mechanism ensures that extracellular enzymes are secreted only at pH values at which they can function effectively. For example, acid and alkaline Pi-repressible phosphatases are secreted into the growth medium at acid and alkaline pH, respectively (14,19). However, there is increasing evidence indicating that *N. crassa* secretes similar amounts of the *pho*-2-encoded Pi-repressible APase irrespective of ambient pH when wild-type strains grow in low-Pi medium, i.e., gene *pho*-2, which is responsive to Pi starvation via a hierarchical regulatory circuit (20), is not an alkaline gene. Interestingly, the synthesis and secretion of an APase is apparently non-responsive to Pi starvation in the dermatophyte *T. rubrum*, since the synthesis and secretion of the APase occurs constitutively (Table 2). In addition, its sensitivity to EDTA (Fig. 2) and dependence on Mg^{2+} ions are properties related to the constitutive APase synthesized, but not secreted, by *N. crassa* (18). The *pho*-2-encoded Pi-repressible APase secreted by *N. crassa* is activated by EDTA, and therefore is not dependent on cations for its catalytic activity (24).

In conclusion, the above results suggest that the secretion of an APase by *T. rubrum* not only relies on independent Pi signaling mechanisms (11), but is also apparently the only APase secreted by this dermatophyte. On the other hand, the very low levels of this phosphatase retained by the mycelium grown on buffered medium at pH 5.0-5.2 (Table 2) lead us to believe that this enzyme is encoded by an alkaline gene. Dermatophytes grow logarithmically in the initial stages of infection, derepressing nonspecific proteolytic enzymes, among others, with optimum activity at acid pH whenever a carbon, nitrogen or sulfur source is lacking in the environment, probably because human skin has a weakly acidic pH (10). It is noteworthy that proteinases with an optimal activity under acidic conditions are important virulence factors in the dermatophytes (25). These proteases act on keratinous and non-keratinous substrates in the stratum corneum, releasing peptides that are hydrolyzed to amino acids by putative peptidases. According to Apodaca and McKerrow (2), the amino acids released in this process may repress the synthesis of nonspecific proteases and may induce the synthesis of keratinases. However, the utilization of some amino acids by fungi that generate cytoplasmic acetyl CoA, such as glycine, for example (Table 1), promotes alkalinization of the growth medium raising

the pH up to values as high as 9.0 (23), an ambient in which most of the known keratinolytic proteases have optimal enzymatic activity (7). Alkalinization predominates over glucose or related sugar-induced acidification of the extracellular medium when the fungi grow on a mixture of glucose and carbon sources generating cytoplasmic acetyl CoA such as acetate or glycine (15,23). Thus, it is possible that the secretion of this APase that is not responsive to Pi starvation is necessary for the dermatophyte to complete its installation, to develop, and to remain in the host (5,6).

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RESUMO

O dermatófito *Trichophyton rubrum* secreta uma fosfatase alcalina EDTA-sensível em meio contendo alta concentração de fosfato

Nesta comunicação nós mostramos que o crescimento do isolado H6 do dermatófito *T. rubrum* em meio não tamponado e sob condição saturante de fosfato, é dependente do pH inicial de cultivo, com um ótimo aparente em pH 4,0. Além disto, independente do pH inicial, o pH do meio se altera durante o cultivo alcançando valores que variam de 8,3 a 8,9. Verificou-se também que este isolado sintetiza e secreta quase os mesmos níveis de fosfatase alcalina, com um ótimo de atividade aparente entre os valores de pH 9,0 e 10,0, independentemente da concentração de fosfato no meio. Também mostramos que essa fosfatase alcalina é inibida por EDTA e ativada por Mg^{2+}. Por outro lado, o nível dessa enzima retida no micélio cultivado em meio tamponado em pH 5,0-5,2 é baixo, sugerindo que ela seja codificada por um gene alcalino, isto é, um gene responsivo à sinalização pelo pH ambiente.

Palavras-chave: dermatófitos, fosfatase alcalina, pH ambiente, regulação fosfato, secreção enzimática, *Trichophyton rubrum*

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