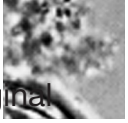

Laccase activity in *Cryptococcus gattii* strains isolated from goats

Eidi Alvarado-Ramírez[1], Josep M. Torres-Rodríguez[1], Maite Sellart[1] and Valerio Vidotto[2]

[1]Research Unit on Infectious Diseases and Mycology (URMIM), Municipal Institute for Medical Research (IMIM), Autonomous University of Barcelona (UAB), Barcelona, Spain; [2]Laboratorio Micologia Medica, Dipartimento Discipline Medico-Chirurgiche, Sezione Malattie Infettive, Universita di Torino Corso, Turin, Italy

Summary

Cryptococcosis is a life-threatening infection in humans and animals caused by encapsulated yeasts of the genus *Cryptococcus. Cryptococcus neoformans* and *Cryptococcus gattii* are the main agents of this mycosis Until 2002 *C. gattii* was classified as a variety of *C. neoformans* but now is accepted as an independent species. The laccase (phenoloxydase) enzyme produced by these yeasts is considered one of the main pathogenic factors for its ability to induce melanin from dihydroxyphenolic compounds. The vast majority of the studies in laccase and melanin synthesis have been developed using isolates of *C. neoformans*. The main objective of this study was to evaluate laccase activity in strains of *C. gattii,* serotype B isolated from immunocompetent goats that died of lung and disseminated cryptococcosis, in several outbreaks occurring in Spain. The laccase activities of these isolates were compared with those of other strains of *C. gattii* and *C. neoformans*. After fungal cell rupture, the supernatant of each isolate was analyzed for its laccase activity using as substrate an L-dopa 20 mM solution. The degree of enzymatic activity was assessed according to its absorbance at 450 nm and scored using Enzymatic Units (EU). The maximum values were observed in three strains of *C. gattii* from goats (EU > 12). The smallest values were observed in one environmental isolate of *C. gattii* serotype C (EU = 0.7). The highest recorded value for *C. neoformans* was 6.3 EU in a serotype A isolate from one human case of meningitis. *C. gattii* serotype B obtained from goats showed different degrees of laccase activity, being the highest in those isolated from severe outbreaks of cryptococcosis. This enzyme appears to represent a major, though nonexclusive, pathogenic factor for *Cryptococcus gattii.*

Key words

Laccase, *Cryptococcus gattii, Cryptococcus neoformans*, Pathogenic factor, Serotypes

Actividad lacasa en cepas de *Cryptococcus gattii* aisladas de cabras

Resumen

Cryptococcus neoformans y *Cryptococcus gattii* son los principales agentes de criptococosis, una grave micosis del hombre y los animales. Entre los factores de patogenicidad de estas especies cabe destacar la lacasa (fenoloxidasa), enzima producida por éstas y otras especies fúngicas, que induce la síntesis de melanina a partir de compuestos di-hidroxifenólicos. La gran mayoría de los numerosos estudios sobre la lacasa se han efectuado con *C. neoformans,* y la información específica en *C. gattii* es muy escasa. El objetivo de este estudio ha sido evaluar la actividad de la lacasa en aislamientos de *C. gattii* serotipo B procedentes de cabras inmunocompetentes muertas por criptococosis durante varios brotes epidémicos desarrollados en Cáceres (Extremadura, España). La producción de lacasa de estos aislamientos se ha comparado con la de otros de la misma especie y también con cepas de *C. neoformans.* Se procedió a la ruptura de las levaduras por métodos físicos, y el sobrenadante de cada aislamiento se añadió a una solución 20 mM de L-dopa.

Address for correspondence:
Dr. Eidi Alvarado-Ramírez
Unitat de Recerca en Malalties Infeccioses i Micologia (URMIM)
Institut Municipal de Investigació Médica (IMIM)
Universitat Autónoma de Barcelona
Parc de Recerca Biomèdica de Barcelona
Tel.: +34 933 160 391
Fax: +34 933 160 410
08003 Barcelona, España
E-mail: ealvarado@imim.es

Aceptado para publicación el 21 de abril de 2008

1130-1406/01/10.00 €

La actividad enzimática se midió a través de la absorbancia como unidades enzimáticas (EU) a 450 nm. Los valores máximos de EU se observaron en tres cepas de *C. gattii* aisladas de cabras (EU >12), mientras que el valor mas bajo se observó en una cepa ambiental de *C. gattii* serotipo C (EU = 0,7).
Para *C. neoformans* la mayor actividad lacasa se obtuvo en una cepa del serotipo A aislada en un paciente con meningitis criptocócica.
Todos los aislamientos de *C. gattii* procedentes de los animales muertos en brotes epidémicos mostraron diferentes grados de actividad lacasa.
Esta enzima parece representar un factor de patogenicidad importante, aunque no exclusivo, en esta especie.

Palabras clave Lacasa, *Cryptococcus gattii, Cryptococcus neoformans,* Serotipos, Factor de patogenicidad

Cryptococcosis is a worldwide life-threatening infection in humans and animals caused by the encapsulated yeasts of the genus *Cryptococcus*. The vast majority of patients have underlying immunodepressed conditions, especially AIDS [12,24]. Nevertheless several cases of cryptococcosis have been described in previously healthy persons or animals [1,5,11,13]. Speed and Dunt [18] observed that many infections occurring in healthy hosts were due to *Cryptococcus neoformans* var. *gattii.* This fact has been observed also in animals, sometimes causing outbreaks [5,17] as those occurred in Spain affecting a high number of goats [1,19]. Recently the variety *gattii* has been raised to species level [7], and currently two differentiated species are accepted: *C. neoformans,* with two varieties: *grubii* (serotype A) and *neoformans* (serotype D); and *Cryptococcus gattii* with the serotypes B and C. Most of the studies on virulence factors do not make the distinction between varieties and serotypes, referring almost exclusively to *C. neoformans*. Thus, it is of great interest to carry out individualized studies to find differences and similarities that may exist between two species so closely related. Several properties of *C. neoformans* and *C. gattii* have been considered important pathogenic factors. The ability to synthesize dark pigments when grown in media with phenolic compounds is one of them [26]. Melanin is the main pigment associated with the virulence in *C. neoformans* [9]. Melanin synthesis is catalyzed by a phenoloxydase that has been categorized as laccase on the basis of substrate specificity [2]. Laccase in the isolates of *Cryptococcus* is found attached to the cell-wall [26], and protective effects against antioxidants are attributed to the resulting melanin, lending support to the integrity of the cellular wall, protection from extreme temperatures, interference with antibody-mediated phagocytosis and lymphocyte T response [2,10]. It has also been considered a defense against various microbicide proteins [4]. Moreover, the melanization of *Cryptococcus* reduces their susceptibilities to amphotericin B and caspofungin [21]. Laccase synthesis is regulated genetically, the participation of the CNLAC1 gene have already been demonstrated [25], and more recently five additional genes have been involved [23]. Chemical and biological characteristics of this enzyme have been studied, but practically only *C. neoformans* isolates have been investigated, and there is scant information on the laccase activity of *C. gattii*. *C. gattii* had been considered a species of limited geographic distribution. However, in the last few years, this species has been found in Europe, specifically in the Iberian Peninsula, producing severe infections in previously healthy goats and in one human [1,3].

The goal of this study was to investigate the activity of laccase in six pathogenic isolates of *C. gattii* serotype B, obtained from the necropsy of goats with disseminated cryptococcosis, comparing the activity of this enzyme with those of other isolates of *C. gattii* (n = 4) and *C. neoformans* (n = 4).

Materials and methods

Isolates. A total of 14 strains of *Cryptococcus* were studied. Table 1 shows their origins. The four *C. neoformans* isolates were from HIV-infected patients with meningeal cryptococcosis.

The six *C. gattii* isolates from goats studied in this work, were selectively randomized as representative of the five Spanish outbreaks previously described [19]. Isolates GR53 and GR56 were from the outbreak nº 1 with 12% of clinical prevalence. GR59 was isolated from the outbreak nº 2 with 2% of clinical prevalence. GR48 was from the cryptococcosis outbreak nº 3 with 10% of clinical prevalence. GR50 from the outbreak nº 4 with prevalence not determined and, finally, strain GR52 was from outbreak nº 5 with clinical prevalence 2.5% [19].

Isolates were maintained in the URMIM collection in skimmed milk at –20 ºC until its use, at which time they were grown on Sabouraud agar with chloramphenicol. The serotypes were confirmed by agglutination with antiserum from a Crypto Check kit, Iatron Laboratories (Tokyo, Japan).

Table 1. Serotypes and origin of 14 isolates of *C. gattii* and *C. neoformans* analyzed for laccase activity.

Isolate	Species	Serotype	Origin	Country	Laccase EU*
A146	*C. neoformans*	A	Human CSF	Spain	1.7
A74	*C. neoformans*	A	Human CSF	Spain	6.3
D297	*C. neoformans*	D	Human CSF	Spain	3.0
D12	*C. neoformans*	D	Human CSF	Spain	4.7
B432	*C. gattii*	B	Human CSF	Mexico	2.7
4506	*C. gattii*	B	Environment	Australia	5.7
GR52	*C. gattii*	B	Goat brain	Spain	1.0
GR59	*C. gattii*	B	Goat lung	Spain	2.7
GR48	C. gattii	B	Goat lung	Spain	4.7
GR50	*C. gattii*	B	Goat lung	Spain	12.3
GR53	*C. gattii*	B	Goat lung	Spain	17.7
GR56	*C. gattii*	B	Goat intestine	Spain	21.0
H0058	*C. gattii*	C	Environment	Colombia	0.7
BV19	*C. gattii*	C	Human CSF	Venezuela	2.0

*EU: Enzymatic Units

Cell rupture. The isolates were grown for 48 h at 30 ºC in Sabouraud agar and immediately cultured in McVeigh & Morton liquid medium [15]. Then they were incubated in slow agitation for 24 h at 25 ºC. Subsequently, the cultures were divided into two equal fractions; one was used as control by inactivating the fungal cells at 56 ºC for 60 min. Yeast cells were centrifuged at 6.000 *g* for 10 min at 4 ºC and then were resuspended in sterile PBS for two rinses. A yeast concentration of 10^8 FCU/ml was resuspended in PBS and cell rupture was carried out in a super-mixer agitator (*Lab-line Instruments*, *INC*. USA) with 0.45-0.60 mm ø glass pearls (Sartorius, Germany). A minimum of ten cycles were performed (with intervals of 2 min in cold bath) until it was microscopically confirmed that more than 80% of the cells had ruptured. The mixture was filtered in Whatmann (Nº 1) sterile paper and centrifuged at 20,000 *g* for 20 min at 4 ºC. The supernatant was conserved at 4 ºC until enzyme determination, performed within a maximum of 72 h.

Controls. The supernatants of the ruptured cells that had been previously heat-inactivated were used as negative controls.

Due to the possibility of auto-oxidation of the substrate, a buffer solution of 20 mM L-DOPA-ring-d_3 in a KH_2PO_4-Na_2HPO_4 0.5 M, pH 6.8, was used, without the supernatant, to control the stability of the reactive (control L-Dopa).

Phenoloxydase (Laccase) assay. The method described previously by Prabhakaran et al. for *Mycobacterium leprae* [16] was adapted to *Cryptoccocus* cells and applied twice for reproducibility study. The activity of laccase was measured by the accumulation of the intermediary chromogenic products that are produced during oxidation of the L-Dopa. Three ml of the supernatant of each isolate mixed with 200 µl of L-Dopa 20 mM solution were incubated in slow agitation at 25 ºC for 120 min. Every 10 min from the moment in which both reactives were mixed, 200 µl of solution were taken and deposited in a flat-bottom microplate. The maximum absorbance of the dopachrome was measured at 450 nm by using a spectrophotometer (Labsystems MultiSkan MS, Finland). The enzymatic activity necessary to increase the absorption value by 0.03 in the indicated conditions, at 30 ºC and pH 6.8 was considered as one enzymatic unit (EU) of laccase. For the final calculation of the EU of each strain, the modified formula, based on the proposal by Vidotto et al. [22] was applied:

$(A\ 450_{120\ min} - A\ 450_{0\ min}) / 10^8$ cells/ml = (EU/10^8 cells/ml)

Statistical analysis. The Mann-Whitney U test was applied to evaluate the individual differences between the values obtained with goats' and reference isolates.

Results

The stability control of the substrate showed that L-Dopa auto-oxidation (A_{450} = 0.04) was not produced. Duplicates give similar values with a maximum of ±0.02 of absorbance changes. When the absorbance increased equal to or greater than 0.03 (EU = 1), the development of a pink pigment was observed in the tube containing the mix. These changes were observed in all tested supernatants, but not in the inactivated-yeast control supernatants. The shortest time to observe the laccase activity was found in two *C. gattii* serotype B supernatants, from goats' isolates, both from the same outbreak (GR56 and GR53), which increased their absorbance after 10 minutes of incubation. These strains reach also the highest values of EU. The figure shows the absorbance values of the supernatants from the 14 strains analyzed at 0 min and 120 min. Maximum absorbance values were obtained in three goat isolates of *C. gattii:* GR56, GR53, and GR50 (Table 2). On applying the above-mentioned formula, the values 21.0 EU, 17.7 EU and 12.3 EU were obtained for these strains, respectively (Figure). Two other non-goat isolates showed high enzymatic activity, one clinical isolate of *C. neoformans* serotype A (6.3 EU) and one environmental isolate of *C. gattii* (5.7 EU) but in any case clearly lower than the goat *C. gattii* mentioned strains ($p \geqslant 0.05$). The lowest laccase activity was observed in strain H0058 of *C. gattii*

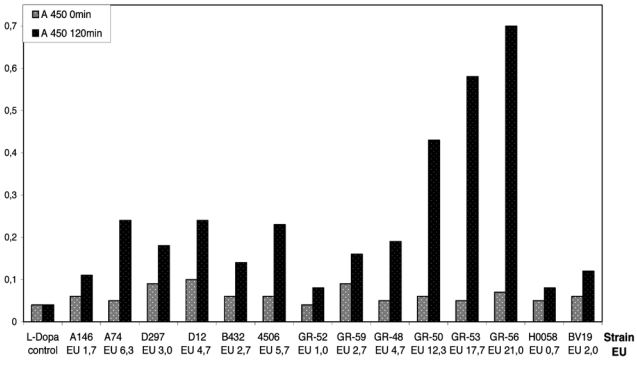

Figure. Absorbance values (450nm) of 14 isolate of *C. gattii* and *C. neoformans* supernatans at 0 and 120 min. One Enzymatic unit (EU) corresponding to absorbance increasing of 0.03.

Table 2. Absorbance values (450nm) of the supernatants from the 14 isolates of *Cryptococcus* spp, analyzed over the course of 0 min to 120 min.

Isolate	Species	0	10	20	30	40	50	60	70	80	90	100	110	120
A146	*C. neoformans*	0.06	0.06	0.06	0.06	0.06	0.07	0.07	0.07	0.08	0.08	0.09	0.11	0.11
A74	*C. neoformans*	0.05	0.06	0.08	0.10	0.11	0.11	0.13	0.15	0.18	0.18	0.24	0.24	0.24
D297	*C. neoformans*	0.09	0.10	0.10	0.11	0.11	0.12	0.12	0.13	0.14	0.14	0.18	0.18	0.18
D12	*C. neoformans*	0.10	0.11	0.12	0.13	0.14	0.15	0.15	0.16	0.16	0.17	0.18	0.24	0.24
B432	*C. gattii*	0.06	0.06	0.07	0.08	0.09	0.10	0.11	0.12	0.12	0.13	0.13	0.14	0.14
4506	*C. gattii*	0.06	0.07	0.08	0.09	0.10	0.12	0.13	0.13	0.15	0.16	0.19	0.23	0.23
GR52	*C. gattii*	0.04	0.04	0.04	0.04	0.05	0.05	0.05	0.06	0.06	0.06	0.07	0.07	0.08
GR59	*C. gattii*	0.09	0.09	0.09	0.09	0.10	0.11	0.11	0.12	0.12	0.13	0.14	0.16	0.16
GR48	*C. gattii*	0.05	0.05	0.07	0.08	0.09	0.10	0.12	0.12	0.14	0.14	0.15	0.17	0.19
GR50	*C. gattii*	0.06	0.06	0.07	0.10	0.15	0.20	0.27	0.30	0.35	0.38	0.41	0.43	0.43
GR53	*C. gattii*	0.05	0.08	0.13	0.18	0.23	0.29	0.32	0.35	0.38	0.42	0.47	0.57	0.58
GR56	*C. gattii*	0.07	0.11	0.16	0.22	0.28	0.34	0.39	0.43	0.48	0.52	0.65	0.70	0.70
H0058	*C. gattii*	0.05	0.05	0.05	0.05	0.06	0.05	0.05	0.05	0.06	0.06	0.06	0.07	0.08
BV19	*C. gattii*	0.06	0.06	0.07	0.07	0.08	0.08	0.09	0.09	0.09	0.10	0.10	0.11	0.12
SCD*	–	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04

*SCD: Stability control of L-Dopa.

serotype C isolate from environmental sources in Colombia and also in one *C. gattii* strain (GR52) isolated from a goat of the less severe outbreak of cryptococcosis.

Discussion

Melanin production has been implicated in *C. neoformans* virulence since the 1980s when it was confirmed that non-melanized mutant strains presented attenuated virulence when they were inoculated in mice, in comparison with wild melanin-containing isolates [8,9]. Practically all of the studies on the features of phenoloxidase activity in *Cryptococcus* have been carried out on *C. neoformans* species, in many cases without specifying either variety or serotype; nevertheless, in 1979, Nurudeen and Ahearn [14] observed that the substances regulating the enzyme correlated with serotype. Ikeda et al. [6], studying several *Cryptococcus* species different to *C. neoformans,* in an epinephrine medium, detected a laccase isoenzyme in ruptured cells of *Cryptococcus albidus, Cryptococcus laurentii* and *Cryptococcus curvatus*, demonstrating that this enzyme is not exclusive to *C. neoformans* and is a variable factor for other *Cryptococcus* species. In the present study, it was shown that three of the pathogenic strains of *C. gattii* serotype B (GR56, GR53, GR50) that led to the highest epidemic outbreaks of cryptococcosis in healthy goats in Spain [1,20], also presented the highest levels of laccase activity in the first 30 min of incubation. Nevertheless, one of the goat strains isolated from a brain tissue sample (GR52) showed very low activity of this enzyme (1 EU). Both isolates of *C. gattii* serotype C also presented low phenoloxidase activity, particularly the environmental isolate sample (H0058). In an earlier study, this strain showed lower pathogenicity when it was inoculated intraperitoneally in immunocompetent mice [20].

The analysis of the results given by *C. gattii* serotype B strains of distinct goats with cryptococcosis, showed that the two isolates that showed a high degree of laccase activity were isolated from animals coming from the epidemic outbreak with the higher clinical prevalence of cryptococcosis (12.5%). By contrast, the isolate GR52 presenting the lowest value of laccase activity was isolated from the outbreak with reduced number of clinical prevalence (2.5%).

The laccase activity of *C. gattii* is variable and it must be considered that the enzyme should be one of the main pathogenicity factors, produced by this fungus but probably not the only one. Further studies need to be carried out in order to determine whether the chemical and genetic characteristics of laccase present in *C. gattii* are different from those in *C. neoformans.*

This study was supported by grant PI02/0413 from Fondo de Investigaciones Sanitarias (FIS), Spanish Ministry of Health. We thank Josep M. Manresa for statistical analysis.

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