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Note

Enzymatic variability among Brazilian *Pythium insidiosum* isolates

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ABSTRACT

Background: *Pythium insidiosum* is an oomycete classified in the kingdom Stramenopila. *P. insidiosum* hyphae are not able to initiate infection without the secretion of hydrolytic enzymes, which are considered an important factor in microbial virulence.
Aims: To evaluate the extracellular enzymatic activity of 14 Brazilian *P. insidiosum* isolates and a standard strain (ATCC 58637) by the API-ZYM System screening method.
Methods: Zoospores were grown in RPMI 1640 broth, and 65 μL of the liquid phase were inoculated in each cupule of the API-ZYM strips.
Results: Differences in the enzymatic activities were observed among the isolates, although phosphohydrolases and ester hydrolases were conspicuous among all isolates. β-glucosidase was also present in most of the isolates. Enzymatic activities of α-glucosidase and chymotrypsin were not observed, differing from a previous study involving Australian isolates and intracellular enzymes.
Conclusions: The discrepancy in the enzymatic profile observed among Brazilian *P. insidiosum* isolates reflects the phenotypic variations found in susceptibility tests.

Variabilidad enzimática entre aislamientos de *Pythium insidiosum en* Brasil

Palabras clave:
Pitiosis
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Exoenzimas
API-ZYM®

RESUMEN

Antecedentes: *Pythium insidiosum* es un patógeno clasificado en el Reino Stramenopila, phylum Oomycota, clase Oomycetes, familia Pythiaceae. Sus hifas no son capaces de iniciar una infección sin la secreción de enzimas hidrolíticas, que se consideran un importante factor de virulencia microbiana.
Objetivos: Examinar la actividad enzimática extracelular de 14 aislamientos de *P. insidiosum* en Brasil y de una cepa de control (ATCC 58637) mediante el método de cribado API-ZYM®.
Métodos: Se cultivaron las zoosporas en caldo RPMI 1640, y se inocularon 65 μL de la fase líquida de las suspensiones celulares en cada cúpula de las galerías del método API-ZYM®.
Resultados: Entre los aislamientos se observaron diferencias en las actividades enzimáticas, aunque las fosfohidrolasas y éster hidrolasas fueron evidentes en todos los aislamientos. La enzima β-glucosidasa también estaba presente en la mayoría de los aislamientos. No se detectaron actividades enzimáticas de α-glucosidasa y quimotripsina, a diferencia de lo observado en un estudio previo efectuado en Australia sobre aislamientos y enzimas intracelulares.
Conclusiones: La discrepancia observada en el perfil enzimático entre aislamientos de *P. insidiosum* en Brasil refleja las variaciones fenotípicas identificadas en las pruebas de sensibilidad.

Among all *Pythium* species, *P. insidiosum* is the only one known to be pathogenic for mammals, with the greatest number of outbreaks occurring in horses, dogs and humans.[6,9,14] This oomycetous pathogen of the kingdom Stramenopila, phylum Oomycota, develops hyphae similar to those found in true fungi. The prevailing model for infection in pythiosis suggests that the infectious propagule is an aquatic zoospore.[9,10] Hyphae emitted towards the host species colonize cutaneous and subcutaneous tissues, produce intestinal lesions, invade blood vessels and can proliferate within bone.

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Although the exertion of invasive pressure is crucial for tissue colonization, hyphae of *Pythium* species are not sufficiently powerful to penetrate solid tissues without the concerted activity of secreted enzymes.[12] It is known that extracellular enzymes, proteases and phospholipases can destroy host tissue and aid fungal invasion. In the recent literature, Davis et al.[4] reported substantial secreted protease activity (serine proteases) in three different *P. insidiosum* strains. The experiments indicated that tissue-degrading enzymes must exert a significant reduction in tissue strength to allow hyphal penetration of plant or animal tissues. As further data on the enzymatic profile of this oomycete and its intraspecific variability with regard to enzyme activity are scarce, we investigated the production of 19 hydrolytic enzymes by Brazilian *P. insidiosum* strains isolated from animal pythiosis.

Materials and methods

This study included twelve Brazilian *P. insidiosum* strains obtained from equine pythiosis, two strains (C20, C28) re-isolated from experimentally infected rabbits (strain CBS 101555)[11] and a culture collection strain (ATCC 58637). The identities of the isolates were confirmed by a PCR-based assay.[1] Strains were initially cultivated in Corn Meal Agar at 37 °C and subsequently subjected to zoosporogenesis.[11,13] Zoospores were counted using a Neubauer chamber and 2.5 to 7.5 × 10^3 zoospores were transferred to 2 mL of RPMI 1640 broth containing L-glutamine (Sigma–Aldrich, Germany) and buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid. Tubes with inocula were maintained at 37 °C for 24 h.

Enzymatic activities were determined by means of the API-ZYM System (bioMérieux, Marcy- l'Etoile, France) in accordance with the manufacturer's instructions. Briefly, 65 μL of the liquid phases from incubated cultures were added to each well of the APY-ZYM tray and maintained at 37 °C for 4 h. Hyphae of *P. insidiosum* were carefully separated from the inoculum. The results were determined in nanomoles (nmol) of the hydrolyzed substrate according to the intensity of the colour reaction on a scale of 1–5, i.e., 1 = 5 nmol, 2 = 10 nmol, 3 = 20 nmol, 4 = 30 nmol and 5 = >40 nmol. The tests were carried out in triplicate and medians were submitted to Kruskal–Wallis statistic test using a significance level of 5%. Differences between the enzymatic activities of all strains and the control group (RPMI broth only) and differences among the isolates were analyzed by the Dunn's multiple comparison test.

Results

The most conspicuous enzymatic activities shown by the 15 *P. insidiosum* isolates were alkaline phosphatase (ALP), esterase, esterase lipase, acid phosphatase (ACP), naphthol-AS-BI-phosphohydrolase and β-glucosidase ($P < 0.01$). Lipase activity was also observed ($P < 0.05$). Eight isolates also produced low levels of trypsin, but the activity of this enzyme did not differ significantly from the control ($P > 0.05$). Within the isolates, differences in the production of β-glucosidase ($P < 0.001$), esterase, naphthol-AS-BI-phosphohydrolase ($P < 0.01$) and ACP ($P < 0.05$) were observed. Data are shown in Table 1.

Our results demonstrate a high intraspecific variability in enzymatic activity. ALP and naphthol-AS-BI-phosphohydrolase activities were detected in 100% of the *P. insidiosum* isolates, whereas esterase and ACP were detected in 93% of the isolates. Esterase lipase, lipase and β-glucosidase activities were detected in 73%, 66% and 60% of the isolates, respectively. No differences in the enzymatic activity were observed according to the animal species from which the strain was isolated (equine or rabbit).

Discussion

The API-ZYM system is a semiquantitative method that permits the rapid detection of 19 enzymes. Many studies have confirmed its usefulness in mycology.[5,16,17] Besides the excellent growth of zoospores, the RPMI broth allowed the establishment of a standardized inoculum; we were also able to analyze the enzymes secreted at the moment that a germ tube is produced by the zoospore, as it occurs in infections.[8] Moreover, bacterial contamination is easily avoided by using this broth, a rather trivial point when an enzymatic profile is required.

Differences in the enzymatic production observed among the isolates were expected. Biochemical variability or inconsistency among Brazilian *P. insidiosum* strains have been reported in susceptibility tests with the same isolates assayed in our study.[2,11] Using western immunoblot analysis, Leal et al.[8] observed important antigenic differences among Brazilian *P. insidiosum* field isolates. However, a more extensive database is required before a key based on phenotypic patterns can be constructed.

Shipton,[15] in an effort to differentiate plant and animal isolates, found similar enzymatic profile from our study for the Australian equine derived *Pythium* spp., with the exception of the presence of α-glucosidase and chymotrypsin. However, instead of zoospores the author used sonicated proteins obtained from hyphal fragments grown on a glucose broth medium (35 °C, 12 h) for inoculating the API-ZYM tray. Based on these observations, two possible hypotheses could explain the differences in the enzymatic profiles: (1) alpha-glucosidase and chymotrypsin are specific intracellular enzymes, because only secreted enzymes were researched in our study; (2) each group studied has a specific enzymatic pattern, according to the geographic cluster in which it is included.[14]

Cellulases (i.e., β-glucosidase in our study) might not be particularly important in pathogenesis, since extensive cellulose degradation typically occurs only late in infection. Notwithstanding, the enzymatic action of β-glucosidase is likely to be fundamental in wall-loosening and growth due to the hydrolysis of β(1–3)-D-glucan linkages present in the *P. insidiosum* cell wall.[3,10]

The expression of phosphatases is ubiquitous among many pathogenic fungi.[16,17] A highly significant positive correlation is established between the adhesion of *Candida* to mammalian buccal epithelial cells and ALP and ACP activity. This relationship, described for the first time by Fernanado et al.,[5] implies that the phosphatases of *Candida* species may play a crucial role in potentiating their virulence.

The ability of the *P. insidiosum* zoospores to initiate an infection is due to the production of a substance that allows them to maintain tight contact with the host.[9] Esterases and lipases both hydrolyze ester bonds. Whereas the lipases display high activity towards the aggregated state of its substrate, the esterases typically show highest activity towards the soluble state of its substrate.[7] Shipton[15] observed lipase activity in all ($n = 14$) Australian equine-derived *Pythium* spp., although only one out of four plant isolates showed activity of this enzyme. Our results suggest that the expressive production of esterases and lipases by *P. insidiosum* aid encystment due to the reduction in surface hydrophobicity.

In conclusion, *P. insidiosum* zoospores grown in RPMI 1640 broth produce extracellular enzymes that diverge among the isolates. In contrast with a previous study, lack of chymotrypsin and α-glucosidase activities was observed. This might be related to genetically distinct populations of *P. insidiosum* strains used in both studies or to the technique employed, since only secreted enzymes were researched in our study. Further enzymatic studies on *P. insidiosum* isolates and non-mammalian pathogenic *Pythium* species, along with the biochemical characterization and purification of the enzymes observed in our study, would enhance the paper of each enzyme in the pathogenic process.

Table 1
Median values ($n = 3$) for the enzymatic activity of 15 *Pythium insidiosum* isolates obtained from horses (Lapemi 129–Lapemi 223), experimentally infected rabbits (Lapemi C20 and C28) and a culture collection strain (ATCC 58637).

Isolates and parameters	Alkaline phosphatase**	Esterase**,†	Esterase lipase**	Lipase*	Acid phosphatase**,†	Naphthol-AS-BI-phosphohydrolase**,†	β-Glucosidase**,†
Lapemi 129	1	3	2	1	1	2	0
Lapemi 138	2	4	3	2	4	2	1
Lapemi 142	2	2	1	1	2	2	5
Lapemi 148	1	2	2	1	2	2	1
Lapemi 152	1	1	0	0	1	1	0
Lapemi 178	1	1	0	0	0	1	0
Lapemi 187	2	2	1	2	2	3	3
Lapemi 205	3	2	3	1	3	2	2
Lapemi 210	2	3	1	1	1	1	2
Lapemi 216	3	3	2	2	2	2	3
Lapemi 220	1	2	2	1	2	2	0
Lapemi 223	2	0	0	0	3	3	0
Lapemi C20	2	3	0	0	2	2	1
Lapemi C28	3	2	1	0	3	2	4
ATCC 58637	2	1	2	1	2	1	0
Range	1–3	0–4	0–3	0–2	0–4	1–3	0–5
Median	2	2	1	1	2	2	1
Number of positives	15	14	11	10	14	15	9

* $P < 0.05$.
** $P < 0.01$ compared to control group.
† Enzymatic activity differed among the isolates ($P < 0.05$).

Conflict of interests

None to declare.

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