The Enzymatic Approach of Zygomycosis - Causing Mucorales

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Various oxidases and hydrolytic enzymes were analyzed to investigate the relationship between these enzymes and the skin pathogenicity of 18 Mucorales strains. Each strain was cultured in a nutrient medium containing starch as a carbon source. The cells grew quickly and were at a good state of growth after incubation for three days. Oxidase activity was not detected in any strain, whereas *Mucor* spp. including *Mucor racemosus* IFM47053 typically had high alcohol dehydrogenase (ADH) activity and all the strains had catalase activity. The culture filtrate and the cell free extract of each strain were applied to APIZYM test system, which revealed that all the strains examined produced many hydrolytic enzymes both inside and outside their mycelia. In the case of *Absidia corymbifera* strains, lipase activity was comparatively high, and polysaccharide hydrolytic enzymes such as α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase were produced.

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Key Words: Zygomycosis, Alcohol dehydrogenase (ADH), Catalase, Hydrolases

INTRODUCTION

Mucor are filamentous fungi of the class Zygomycetes that are found in soil, plants, decaying fruits and vegetables. These saprophytic fungi grow fast and appear early in the process of material decomposition. Organisms of the *Mucor* species are distributed worldwide. Not only ubiquitous in nature but also a common laboratory contaminant, some Mucorales such as genus *Rhizopus*, *Mucor*, *Absidia*, *Mortierella* may cause the disease mucormycosis[1,2] or zygomycosis. The latter term is becoming common as this disease can be caused by other members of the Zygomycete class of fungi in addition to the *Mucor* species. Most species of *Mucor* are unable to grow at 37℃ but the strains isolated from human infections are usually thermotolerant. A characteristic feature of human zygomycosis is that it causes a fence-sitting infectious disease by weakening the immunity of a host.

The role of oxidation-reduction enzymes in the skin pathogenicity of Zygomycetes has seldom been investigated. In order to study of the relationship between enzymes and pathogenicity, we measured various oxidizing and hydrolysing enzymes by applying the APIZYM 20 test system to the 18 pathogenic strains of Zygomycetes.

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MATERIAL AND METHODS

1. Organism, medium and culture conditions

Eightteen strains of Mucorales were obtained from Chiba University Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba. The strains (Table 1) were maintained on potato dextrose agar (PDA; Becton Dickinson, Sparks, MD, USA). The medium (liquid medium) for the production of the oxidoreductases contained the following compounds (g per liter): starch, 20; $NaNO_3$, 5; K_2HPO_4, 5; $MgSO_4 \cdot 7H_2O$, 1; yeast extract, 0.2; trace salt solution, 2 ml; vitamin solution, 0.2 ml. The trace salt solution had the following composition (g per 100 ml): $FeC_6H_5O_7 \cdot nH_2O$, 0.6; $CaCl_2 \cdot 2H_2O$, 0.4; $ZnSO_4 \cdot 7H_2O$, 0.2; $MnCl_2 \cdot 4H_2O$, 0.01; KI, 0.01; $6(NH_4)\ Mo_7O_{24} \cdot 4H_2O$, 0.01; $CoCl_2 \cdot 6H_2O$, 0.01; H_3BO_4, 0.01; NaCl, 0.5. The vitamin solution had the following composition (g per 100 ml): biotin, 0.002; Ca-pantothenate, 0.4; pyridoxin-HCl, 0.4; p-aminobenzoate, 0.2. The medium (150 ml) in a 500-ml Erlenmeyer flask was sterilized by autoclaving at 121℃ for 20 min. The flask was inoculated and incubated at 30℃ on a rotary shaker (180 rpm). After incubation for four days, cells were collected by centrifugating the culture. The resultant cells were washed twice with 50 mM potassium phosphate buffer (pH 7.2) and stored at -20℃ until use.

Table 1. Strains belonged to Mucorales used in the present experiments

Strain
Mucor circinelloides IFM47052
Mucor ramosissiumus IFM46006
Mucor circinelloides IFM46508
Mucor racemosus IFM40781
Mucor racemosus IFM47053
Mucor hiemalis IFM41559
Cunninghamella bertholletiae IFM46110
Cunninghamella bertholletiae IFM46111
Cunninghamella elegans IFM46109
Cunninghamella elegans IFM47050
Rhizopus oryzae IFM40515
Absidia corymbifera IFM41345
Absidia corymbifera IFM47048
Absidia corymbifera IFM5335
Absidia corymbifera IFM40776
Absidia cylindrospora IFM40501
Absidia cylindrospora IFM40777
Absidia glauca IFM41346

2. Enzyme assays

1) Alcohol oxidase activity

Alcohol oxidase activity was assayed by measuring oxygen consumption using an oxygen electrode (Oxygraph 9; Central Science, Tokyo, Japan)[3]. The incubation cell (1 ml) contained 100 μmol of alcohol, 100 μmol of potassium phosphate buffer (pH 7.2) and 100 μl of enzyme preparation, and it was incubated at 30℃. One unit (U) of alcohol oxidase was defined as the amount of enzyme that consumed 1 μmol of oxygen per min.

2) Catalase acitivity

Catalase activity was measured in cell free extract and culture filtrate by the method of Aebi[4]. The decomposition of the substrate H_2O_2 was monitored spectrophotometrically at 240 nm. Specific activity was defined as the micromole substrate decomposed per minute per milligram of protein (i.e. U/mg protein).

3) Dehydrogenase activity

Enzyme assays were carried out in a 1 ml volume with 50 μM β-NAD^+ cofactor, 1 M substrate (alcohol, lactate, malate, mannitol, alanine etc.) and they were monitored for reduction of the cofactor at 340 nm, $e_{340} = 6220\ M^{-1}\ cm^{-1}$ [5]. The spectrophotometer used was a Shimadzu UV-2500 (Kyoto, Japan). For the enzyme kinetic characterization,

50 mM potassium phosphate buffer was used at pH 7.4 at a temperature of 30℃.

4) Laccase activity

Laccase activity was determined by measuring the oxidation of 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) at 420 nm ($\varepsilon = 3.6 \times 10^4$ cm^{-1} M^{-1})[6]. The reaction mixture contained 0.03% ABTS, 0.1 M sodium acetate buffer, pH 5.0 and a suitable amount of enzyme solution. One laccase activity unit was defined, as the amount of enzyme that oxidized 1 μmol ABTS per min.

5) Hydrolase activity APIZYM application

Cell free extract and culture filtrate of each strain were inoculated into the APIZYM test system, incubated for 4 h at 30℃ and processed according to the manufacturer's directions[7] in order to determine the strain's enzyme profile. The APIZYM system (BioMerieux Vitek) consists of 19 hydrolase substrates, alkaline phosphatase, esterase, esterase lipase, lipase, leucin arylamidase, valine arylamidase, cysteine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphtol-AS-BI-phosphohydrolase, alpha-galactosidase, beta-galactosidase, alpha-glucosidase, beta-glucosidase, N-acetyl-beta glucosaminidase, alpha-mannosidase, and alpha-fucosidase. APIZYM allows the rapid detection of constitutive enzymes based on a colorimetric assay. The intensity of color development following the addition of reagents, determines the presence and level of enzyme activity.

3. Protein assay

Protein was measured by the method of Bradford[8] with bovine serum albumin as the standard protein.

4. Chemicals

The other reagents which are not referred to specifically are from Wako Pure Chemical (Osaka).

RESULTS

1. The growth of Zygomycetes

Each Zygomycete was grown in the medium composition and under the culture conditions described above. Genus *Cunninghamella* and *Mucor circinelloides* demonstrated fast growth, approaching the maximum mycelia production after incubation for three days. The mycelia production of genus *Absidia* was quite good, but the level achieved after incubation for two days was maintained without further increasing. *Mucor ramosissimus* grew very slowly and their number of mycelia also increased slowly. *Rhizopus oryzae* showed poor mycelia production with a spool-like form.

2. Oxido-reductase production

1) Oxidase activity

Various cell free extracts from 18 strains of Zygomycetes were screened using Oxigraph, to detect oxidase activity against each substrate (glucose, methanol, ethanol, mannose, mannitol, guaiacol, glycin, maleic acid, nicotinamide, lactose, tyrosine, inositol, lysine, xylose, fructose, glucosamin and arabinose). No oxidase activity was demonstrated for any of the 18 strains (data not shown). Moreover, no trace of laccase was detected in the cell free extract. Therefore we conclude that the Zygomycetes do not produce oxidases as their main enzymes.

2) Catalase activity

Catalase activity was assessed in the cell free extract of the Zygomycetes. All the Zygomycetes had some degree of catalase activity. Genus *Cunninghamella* showed the most abundant catalase production (Table 2). *Mucor* spp. also had some catalase production in their culture filtrates (data not shown).

3) Dehydrogenase activity

Various dehydrogenase activities were screened

Table 2. Catalase and alcohol dehydrogenase activity by various strains of Mucorales

Strain	Catalase (U/mg)	ADH (U/mg)
M. circinelloides IFM47052	148	0.53
M. ramosissiumus IFM46006	133	0
M. circinelloides IFM46508	139	0.64
M. racemosus IFM40781	157	0.034
M. racemosus IFM47053	143	1.022
M. hiemalis IFM41559	235	0.50
C. bertholletiae IFM46110	94	0
C. bertholletiae IFM46111	57	0
C. elegans IFM46109	562	0
C. elegans IFM47050	214	0
R. oryzae IFM40515	– [a]	–
A. corymbifera IFM41345	235	0
A. corymbifera IFM47048	221	0.05
A. corymbifera IFM5335	141	0
A. corymbifera IFM40776	173	0.02
A. cylindrospora IFM40501	45	0.31
A. cylindrospora IFM40777	158	0.04
A. glauca IFM41346	451	0.11

[a], The strain could not be assayed because of the poor growt

using the cell free extract of the Zygomycetes. NAD^+ was used as a cofactor in the reaction mixture and the reaction was started with the injection of the substrates ethanol, lactate, malate, alanin, and mannitol. *Mucor* spp. in particular demonstrated vigorous alcohol dehydrogenase activity (ADH) (Table 2). The ADH activity pattern for the *Mucor* spp., *M. racemosus* 47053, *M. circinelloide* 46508, *M. circinelloides* 47052 and *M. hiemalis* 41559, is shown in Fig. 1. Other Zygomycetes did not have any dehydrogenase activity.

3. Hydrolase activity (APIZYM)

After incubating for four days, cell free extracts were applied to the APIZYM 20 test system in order to examine the production of hydrolases by 18 Zygomycete strains (Table 3). All strains had high phosphatase activity, both alkaline phosphatase and acid phosphatase, and arylamidase activity, including leucine arylamidase and valine arylamidase. Additionally, esterase, naptol-AS-BI-phosphohydrolase and alpha-mannosidase enzymes were common in the tested Zygomycetes. *Absidia* spp. were demonstrated that they produce distinguished polysaccharide hydrolytic enzymes (alpha-glucosidase, beta-glucosidase, N-acetyl-beta-glucosaminidase, alpha-mannosidase, alpha-fucosidase, etc.), and *Absidia corymbifera* had the highest lipase activity. The culture filtrate after incubation for four days was also applied to the APIZYM test system and the results are shown in Table 4.

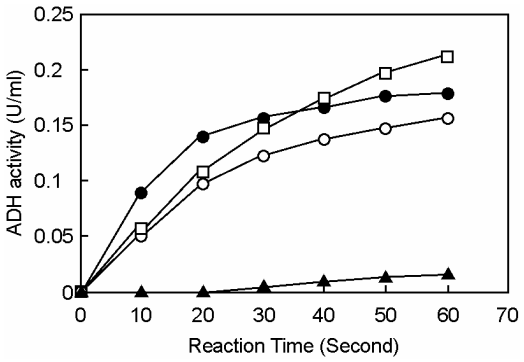

Fig. 1. Alcohol dehydrogenase activity of *Mucor* spp. ○, *Mucor circinelloides* IFM47052; ●, *Mucor circinelloides* IFM46508; □, *Mucor racemosus* IFM47053; ▲, *Mucor hiemalis* IFM41559.

DISCUSSION

Mucorales are widespread environmental fungi which live in decaying vegetables, fruits, seeds, and air[9]. The opportunistic Mucorales infections in humans are called mucormycosis, and such infections generally lead to the death of patient[10]. These harmful human infections have been increasingly reported in transplant recipients, and in patients with diabetes mellitus, HIV infection, cancer, hema-

Table 3. APIZYM application for cell free extract of Mucorales

Enzyme Number[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Strains																				
M. circinelloides IFM47052		5[b]	5	4		5	4			4	5	5	2	1		3	2		5	1
M. circinelloides IFM46508		5	4	3		4	4			4	5	5	2			2	2		5	
M. ramosissimus IFM46006		5	5	4		5	4			2	3	5	1				1	1	1	
M. racemosus IFM40781		5	4	4	1	4	4			3	5	5						4		1
M. racemosus IFM47053		5	4	4	2	4	4			4	5	5				1	3	5	3	
M. himalis IFM41559		5	2			4	1			1	5	5					1		2	
C. bertholletiae IFM46110		5	5	4		5	4			1	5	5						2	5	
C. bertholletiae IFM46111		5	4	2		4	3			1	5	5						1	1	
C. *elegans* IFM46109		5	4	2		5	3			2	5	5				2			5	
C. elegans IFM47050		5	4	2		5	4				4	5							5	
R. oryzae IFM40515		5	5	2		5	3		3		5	5	5				5		5	
A. corymbifera IFM41345		5	5	5	5	5	5	1		1	5	5				3	1	4	5	2
A. corymbifera IFM47048		5	4	5	5	5	5				5	5				2		2	5	1
A. corymbifera IFM5335		5	4	4	2	4	4	2		4	5	5	2			2	5	5	5	
A. corymbifera IFM40776		5	4	4	2	4	4	2	1	4	5	5	2			2	5	5	5	
A. cylindrospora IFM40501		5	3	2		4	3			3	5	5	2			5	5	5	3	
A. cylindrospora IFM40777		5	3	3	1	4	4			4	5	5	2			1	5	5	5	
A. glauca IFM41346		5	2	2		4	3			2	5	5						1	1	

[a] Enzyme number: 1, control; 2, alkaline phosphatase; 3, esterase; 4, esterase lipase; 5, lipase; 6, leucin arylamidase; 7, valine arylamidase; 8, cysteine arylamidase; 9, trypsin; 10, chymotrypsin; 11, acid phosphatase; 12, naphtol-AS-BI-phosphohydrolase; 13, alpha-galactosidase; 14, beta-galactosidase; 15, alpha-glucuronidase, 16, alpha-glucosidase; 17, beta-glucosidase; 18, N-acetyl-beta glucosaminidase; 19, alpha-mannosidase; 20, alpha-fucosidase.
[b] The color scale 1 to 3 means enzyme activity ranged from low to moderate, and 4 to 5 means high enzyme activity

Table 4. APIZYM application for culture filtration of Mucorales

Enzyme Number[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Strains																				
M. circinelloides IFM47052		1[b]	3	2		4														
M. circinelloides IFM46508						3						4				2	2			
M. ramosissimus IFM46006		2	2	2		5					1	1								
M. racemosus IFM40781		3		2	1	4					5	5					4			
M. racemosus IFM47053						4						3						2		
M. himalis IFM41559		4										2								
C. bertholletiae IFM46110		1				4														
C. bertholletiae IFM46111		4				4					1	1								
C. elegans IFM46109			1			5					2									
C. elegans IFM47050						3					1	1								
R. oryzae IFM40515		2	2	1		2					1	1	1							
A. corymbifera IFM41345			5	5	3	5					2									
A. corymbifera IFM47048			4	4	3	5					3	1								
A. corymbifera IFM5335		5		3	3	3					5	5					5			
A. corymbifera IFM40776		5		2	1	3					4	4					5			
A. cylindrospora IFM40501				3	1	1	2					2				3	3	5		
A. cylindrospora IFM40777		4		2	1	3	1				5	4					3			
A. glauca IFM41346						2					1	1								

[a] Enzyme number: 1, control; 2, alkaline phosphatase; 3, esterase; 4, esterase lipase; 5, lipase; 6, leucin arylamidase; 7, valine arylamidase; 8, cysteine arylamidase; 9, trypsin; 10, chymotrypsin; 11, acid phosphatase; 12, naphtol-AS-BI-phosphohydrolase; 13, alpha-galactosidase; 14, beta-galactosidase; 15, alpha-glucuronidase, 16, alpha-glucosidase; 17, beta-glucosidase; 18, N-acetyl-beta glucosaminidase; 19, alpha-mannosidase; 20, alpha-fucosidase.
[b] The color scale 1 to 3 means enzyme activity ranged from low to moderate, and 4 to 5 means high enzyme activity

tological diseases, etc.[9,11] Currently, the detection of these genera is usually either at autopsy or from surgical specimens if available, but molecular techniques such as PCR-RFLP[10] could prove useful for fungal identification.

In this study, 18 strains of *Mucor*, *Cunninghamella*, *Rhizopus* and *Absidia* belonging to Mucoraceae were selected and cultured in a liquid medium, and their growth in culture and morphological features were compared. In addition, both culture filtrate and cell free extract of these strains were used for the analysis of oxidoreductive enzymes, and were applied to the APIZYM 20 test system to compare their hydrolase activity patterns.

Rhizopus was distinctive in its poor growth in culture and mycelia formation compared to the other tested Mucorales under same culture conditions. A similar finding was described in the report which classified Mucorales using various carbon sources and found that *Rhizopus* could not be activated on carbon sources such as fructose, glucose, galactose, arabinose and xylose in contrast to other Mucorales[12].

Some *Mucor* strains as *M. ramossimus*, *M. circinelloides* showed characteristic alcohol dehydrogenase activity[13].

Strains belonging to same genus generally produced the same enzyme pattern in the APIZYM test system. *Absidia* spp. produced comparatively high amounts of beta-glucosidase both inside and outside the cells. These findings suggest that the lipase activity and polysaccharide hydrolytic enzymes in genus *Absidia* may be involved in physiological mechanisms and pathogenesis. The modification[14] of glycoprotein, a constituent of cell membranes, may be related to a host infection. Skin pathogenic Zygomycetes in this work showed an abundant amount of hydrolase activity, such as phytase[15] and protease[16], both intracellularly and extracellularly. Some types of lactic acid bacteria have been reported to produce beta-glucosidase, a hydrolytic enzyme harmful to human beings[17]. The use of the APIZYM test system in this research enabled the differentiation of Mucorales species similar to classification based on the similarity of ITS sequence using PCR[18] or carbon source utilization[12]. This research is the first enzymological approach to analyse various oxidoreductases and hydrolases produced by 18 strains of zygomycosis-causing Zygomycetes. The relationship between the pathogenicity of each Mucoraceae and the host cell infection remains for further study.

CONCLUSION

We studied the relationship between skin pathogenic Zygomycetes (18 strains) and their enzyme composition (oxidoreductase: catalase, laccase, alcohol oxidase, ADH, and 19 hydrolases included in the APIZYM test system). 1. Genus *Mucor* had considerable ADH activity and comparatively good catalase activity in both the cell free extract and the culture filtrate. 2. Genus *Absidia* produced catalase, ADH and malate dehydrogenase slightly in each strain. *Absidia* spp. showed high beta-glucosidase activity in the APIZYM application. 3. Genus *Cunninghamella* had higher catalase activity than other genii. 4. The tested Zygomycetes did not produce any oxidase at all. 5. Each strain showed its own characteristic enzyme pattern in the APIZYM test system, with each genus having a similar enzyme pattern.

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= 국문초록 =

Zygomycosis 유발성 Mucorales의 효소학적 특성 연구

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본 연구는 진균증 유발성의 털곰팡이목 균주가 생산하는 각종 효소의 생산성을 조사하여 병원성과의 관련성을 밝히고자 하였다. 치바대학 진균의학연구센터가 보유하고 있는 *Mucor*속, *Cunninghamella*속, *Rhizopus*속, *Absidia*속 등의 18개 균주를 사용하여 배양 배지 상의 생육특성 및 세포 내·외의 각종 산화효소, 카탈라아제 및 가수분해효소의 분포성을 분석하였다.

생육 배지상의 특징으로 Starch를 탄소원으로 한 영양배지에서 *Rhizopus oryzae* IFM40515는 배양 초기 실타래와 같은 모양으로 자라나나, 생육이 매우 느리며 균사의 증가를 거의 보이지 않아 테스트한 다른 Zygomycete 균주들과는 생육면에서 상이한 양상을 보였다.

테스트한 18균주에서 oxidase의 검출은 거의 나타나지 않았으며, *Mucor racemosus* IFM47053을 포함한 *Mucor* spp.는 높은 알코올탈수소효소 활성을 나타내는 특징이 있었다. 카탈라아제는 모든 균주의 세포 내에서 검출 가능하였다. 가수분해효소를 APIZYM을 이용하여 분석한 결과는 세포 내 효소뿐만 아니라 세포 외로 분비되는 효소에 있어서 각 균주의 효소패턴은 같은 속에 속하는 균주의 효소패턴과 유사성을 나타내는 것으로 사료되었다. *Absidia corymbifera* 균주의 특징은 다른 종의 균주와 비교해서 lipase 활성이 매우 높았으며, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase 등의 다당가수분해효소의 활성이 높았고, 특히 β-glucosidase는 세포 외로도 활발히 분비하는 것으로 나타났다.

색인 단어: 진균증, 알코올탈수소효소, 카탈라아제, 가수분해효소