Intracellular Serine Protease from *Candida glabrata* Species Detected and Analyzed by Zymography

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Abstract

To detect the intracellular proteases of *Candida* species, we examined the zymography using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing soluble casein which was a substrate for proteolytic digestion. The cell extracts from three *Candida* species (*C. glabrata*, *C. tropicalis*, and *C. krusei*) showed different banding patterns on casein-containing gels. The extracts derived from four different strains of *C. glabrata* showed same pattern. The proteolytic activity of intracellular protease derived from *C. glabrata* was inhibited by phenylmethylsulfonyl fluoride (PMSF), so this protease is one of serine proteases. The zymography is useful method for detection and characterization of *Candida* species proteases.

Key words: intracellular, protease, *Candida glabrata*, zymography

Introduction

*Candida* species are among the most common fungal pathogens of humans and account for a large proportion of all systemic fungal diseases ¹. Many secreted proteases have been reported as virulence factors in fungal infection or allergy, but little is known about intracellular proteases of fungi. The present study was performed to detect some proteases present within the *Candida* cell and to analyze their characteristics using proteomic methods, “Zymography”²,³. Zymographic techniques allow the detection of proteases following electrophoresis in various types of gel matrix ⁴. Zymography with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was originally reported by Laemmli ⁵ and a modified method was described by Heussen and Dowdle ⁶. The visualization, molecular weight estimation and biochemical characterization of the bacterial proteases have been performed using zymography ⁷. In the present study, we successfully detected non-secreted proteases extracted from major pathogenic *Candida* spp by zymography. To our knowledge, this is the first study of zymography applied to fungal proteases.

Materials and Methods

Organisms and culture conditions

Fresh clinical isolates of *Candida* species used in this study were obtained from Tokyo Women’s University (Table 1). These strains were maintained on solid YPD medium (10 g/l of yeast extract, 20 g/l of peptone, 20 g/l of glucose, 15 g/l of agar) ⁷).
(Millipore, Billerica, MA, U.S.A.) was added to the cell suspension, followed by centrifugation at 20,000 × g for 60 min to remove cell debris, and the obtained supernatant was used as the crude cell extract.

Zymography

Proteases included in the crude yeast cell extracts were analyzed by zymography following SDS-PAGE on 7.5% mini-gels. Zymography was performed as described previously by Heussen and Dowdle with minor modification. Samples were loaded to SDS-PAGE in gels (7.5% acrylamide/0.2% bis-acrylamide) containing 0.2% (w/v) soluble casein (Wako Pure Chemical Industries, Osaka, Japan) without reduction nor boiling, and the runs were carried out at 4 °C using pre-cooled buffers. After electrophoresis, the gels were washed in 2.5% (v/v) Triton X-100 (Sigma, St. Louis, MO, U.S.A.) for 30 min to remove SDS and allow the proteases to renature. The gels were then incubated in phosphate buffer (composition) for 15 h at 37 °C and stained with Coomassie brilliant blue (CBB) R-250. Trypsin solution (Wako) was used as a protease control.

Protease inhibition assay

Ethylenediamine-tetraacetic acid (EDTA) and epoxysuccinyl-leucylamido(4-guanidino)-butane (E64) were dissolved in ultrapure Milli-Q water, pepstatin A was dissolved in acetic acid/methanol solution (1:9) and phenylmethylsulfonyl fluoride (PMSF) and 1,10-phenanthroline were dissolved in methanol. All protease inhibitors were purchased from Sigma, with the exception of EDTA which was from Wako. After SDS-PAGE in 7.5% acrylamide, 0.2% bis-acrylamide gel containing 0.2% soluble casein (Wako) and washing in 2.5% (v/v) Triton X-100 (Sigma), the gels were incubated in enzyme reaction buffer containing each protease inhibitor for 15 h at 37 °C and stained with CBB R-250. The final concentrations of each protease inhibitor included in the enzyme reaction buffer are shown in Table 2.

Results

Detection of proteolytic activities from cell extracts of Candida species by zymography

To detect proteases included in the cells of Candida species using zymography, soluble casein,
as a substrate for proteolytic digestion, was co-polymerized with SDS-PAGE gels. The cell extracts from three Candida species (C. glabrata, C. tropicalis, and C. krusei) showed different banding patterns after proteolytic digestion on the casein-containing gels (Fig. 1). Single intense bands with molecular weights varying from 60 to 80 kDa were detected in the cell extracts from C. glabrata, C. tropicalis, and C. krusei. From comparison with molecular weight standards, the molecular weights of these bands were estimated to be approximately 64 kDa in C. glabrata, 78 kDa in C. tropicalis, and 66 kDa in C. krusei. A single faint band was also detected in the C. tropicalis cell extract. There were no differences in the banding patterns intra-species of C. glabrata (Fig. 2). We also examined the cellular extracts of C. albicans in this assay (data not shown). The cell extracts from C. albicans gave proteolytic bands with lower molecular weights, which overlapped with many polypeptide bands stained by CBB, and were unsuitable for further analysis. Thus, subsequent assays for proteases were carried out using the C. glabrata cell extracts.

### pH optima of proteases from the C. glabrata cell extracts

Zymography with casein-containing gels visualized a single proteolytic band for the C. glabrata cell extract. We designate this protease as Prt-Cg. To determine the effects of pH on the proteolytic activity of Prt-Cg, the casein-containing gels after electrophoresis were incubated in several enzyme reaction buffers with different pH values (pH 5.0-10.0). As shown in Fig. 3, the maximal hydrolytic activity was observed at pH 6.0. Thus, subsequent assay for Prt-Cg was carried out at pH 6.0.

### Classification of Prt-Cg by protease inhibitors

The effects of different protease inhibitors on the proteolytic activity of Prt-Cg were studied using zymography. After electrophoresis, the casein-containing gels were incubated in enzyme reaction buffers with various concentrations of PMSF, E-64, EDTA, and pepstatin A. The results are summarized in Table 2. Of the above inhibitors, only PMSF had an evident inhibitory effect on the hydrolytic activity of Prt-Cg for casein (Fig. 4), which was dose-dependent manner (data not shown). Thus, Prt-Cg was indicated to be a serine protease.

### Discussion

In the present study, the proteolytic activities of cell extracts prepared from three Candida species were examined using zymography coupled with SDS-PAGE. Despite crude preparation, all the cell extracts exhibited evident proteolytic bands on the gels (Fig. 1), indicating that zymography is a sensitive technique for enzymological analyses. Different factors, such as specific enzyme inhibitors and pH, can be introduced into zymography (Fig. 3, Fig. 4, Table 2), facilitating the identification of several biochemical characteristics of target enzymes without requiring isolation. In addition, soluble casein was used as a substrate molecule in this study, although other proteins are also available for this purpose.

The zymography system yielded results for only a few active proteases in Candida cell extracts (Fig. 1). In general, living cells produce a variety of proteases for maintenance of growth, and thus a large number of proteases may be inactivated in the cell extracts.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Inhibition</th>
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<tbody>
<tr>
<td>PMSF</td>
<td>1-10 mM</td>
<td>+</td>
</tr>
<tr>
<td>E-64</td>
<td>1-20 µM</td>
<td>-</td>
</tr>
<tr>
<td>EDTA</td>
<td>1-10 mM</td>
<td>-</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>1-10 µM</td>
<td>-</td>
</tr>
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+ : detectable inhibition, - : no detectable inhibition.

PMSF : Phenylmethylsulfonyl fluoride, E-64: Epoxysuccinyl-leucylamido (4-guanidino)-butane, EDTA: ethylenediamine-tetraacetic acid, Prt-Cg: protease of C. glabrata.
Zymography coupled with SDS-PAGE system was used in this study, as reported previously 5). SDS in the sample buffer and polyacrylamide gels may cause irreversible conformational changes in these proteases, resulting in their inactivation. The results suggested that a native PAGE system would be more suitable for zymography of *Candida* cell extracts, which would also provide additional characteristics of the cell-localized proteases. On the other hand, no proteolytic bands were obtained from the *C. albicans* cell extracts, because the proteolytic signals were overlapped with many polypeptide bands stained with CBB. There is a possibility to more suitable methods of preparation of the *C. albicans* extracts. Zymography can also be coupled with two-dimensional PAGE systems, the use of which may make it possible to separate proteolytic signals from these polypeptide bands.

There have been no previous reports of the isolation or characterization of cell-localized proteases from *C. glabrata*. The proteolytic activity of Prt-Cg, which was detected in the *C. glabrata* cell extracts, was markedly reduced by PMSF (Fig. 4, Table 2), indicating that Prt-Cg is a serine protease 11). Recently, almost the whole genome DNA sequence of *C. glabrata* has been determined 12). We tried to identify Prt-Cg in DDBJ/EMBL/GenBank database. Eight proteases in seven accession number have successfully been identified based on molecular weight and type of active center (Accession number: XP_446588, XP_447240, XP_447258, XP_449152, XP_445129, XP_445375, CAG60220).

Zymography is a sensitive and simple analytical method that will play important roles in post-genomic and comparative phenotypic researches in the field of medical mycology.

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References


**Abbreviations**

CBB: coomassie brilliant blue, E64: epoxysuccinyl-leucylamido(4-guanidino)-butane, EDTA: ethylenediaminetetraacetic acid, PBS: phosphate buffered saline, PMSF: phenylmethylsulfonyl fluoride, Prt-Cg: protease of *C. glabrata*, SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
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Figure 1  Detection of proteolytic activities from cell extracts of *Candida* species by zymography. Soluble casein was co-polymerized with SDS-polyacrylamide gels. Lane 1: Crude extract of *Candida glabrata*. Lane 2: *C. tropicalis*. Lane 3: *C. krusei*. Single intense bands with molecular weights of 60 to 80 kDa.

Figure 2   Intra-species comparison of zymography banding patterns in the *C. glabrata* cell extracts. Lane 1: Trypsin control. Lane 2-5: TWCC 13395, 13405, 13428, 13434.
Figure 3  Effect of pH of incubation buffer. To examine the effect of pH on the activity of proteases included in the C. glabrata cell extract, three kind of enzyme reaction buffers were used: pH 5.0: 50 mM sodium acetate buffer, pH 6.0-7.0: disodium phosphate buffer (0.1 M NaHPO₄-NaH₂PO₄), pH 8.0-10.0: boric acid buffer (50 mM Na₂B₄O₇).

Figure 4  Effect of 5 mM serine protease inhibitor (PMSF) on the proteolytic activity of Prt-Cg. Lane 1: Incubated in phosphate buffer. Lane 2: Incubated in phosphate buffer with 5 mM PMSF.