Short Report

Quantitation of Fungal DNA Contamination in Commercial Zymolyase and Lyticase Used in the Preparation of Fungi

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Abstract

Small amounts of contaminants may lead to false-positive results in sensitive polymerase chain reaction (PCR) detection systems. To analyze contaminants and understand the usability of β -glucanases in fungal preparations, we estimated the ribosomal DNA (rDNA) contamination in Zymolyase-100T and Lyticase by quantitative PCR. The amount of rDNA contamination determined by real-time PCR was 9210 copies/unit for Zymolyase-100T and 0.0323 copies/unit for Lyticase. The observations regarding these enzyme products indicate that careful consideration of contaminating DNA included in the reagents used for molecular diagnostics is necessary.

Key words : β -glucanase, DNA contamination, real-time PCR

INTRODUCTION

Instead of culture or microscopic analysis, molecular biological techniques for environmental or invasive pathogenic fungi based on polymerase chain reaction (PCR) of DNA have been widely applied for clinical investigation¹⁾. Over the past several years, a number of research groups have constructed methods for detection of a wide-range of environmental and pathogenic fungi by fluorescent probe PCR systems²⁾. These sensitive methods can detect a fungal DNA fragment at levels as low as only 1 copy. PCR-based methods have the advantages of high sensitivity and specificity, although small amounts of contaminants may lead to false-positive results in those PCR detection systems^{3, 4)}.

For separation of genomic DNA template used in these PCR methods, enzymatic lysis methods are generally applied to break the fungal cell wall^{5, 6)}. The enzyme used most frequently for digestion of the fungal cell wall is β -glucanase⁷⁾. Highly purified recombinant β -glucanases are now available⁸⁾, but enzymes such as Zymolyase⁹⁾ (developed by Kirin Brewery K.K., supplied commercially by Seikagaku Biobusiness,

Zymo Research, MP Biomedicals, and Geno Technology) or Lyticase¹⁰⁾ (Sigma-Aldrich) are inexpensive and applied widely for genetic applications. Despite its dependability and convenience, several groups have reported fungal ribosomal DNA in these enzyme preparations^{11, 12)}. The effects of such contaminants differ depending on origin, the experimental system used, and the amount of enzyme used in each study. Here, we determined the actual quantity of fungal DNA in two major enzyme preparations using a TaqMan real-time PCR system¹³⁾ to assess the applicability of these enzymes required in many of the molecular diagnostic approaches.

MATERIALS AND METHODS

Construction of positive control: Genomic DNA from the *Aspergillus niger* strain JCM 10253 was collected as described previously for use as the templates¹⁴⁾. The D1/D2 region on *A. niger* 28S rDNA was amplified by PCR¹⁵⁾, and cloned into the plasmid pCR2.1 using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Qubit fluorometer and Quant-iT dsDNA HS Assay Kit (Invitrogen) were used for quantitation of dsDNA in standard plasmid or enzyme preparations. Plasmid copy number was determined by its size and the concentration of dsDNA.

Quantitative PCR: All of the preparations were per-

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Fig. 1 Quantitative PCR amplification curves of standard plasmids and β -glucanase dissolved in pure water as templates. Curves for serial dilutions of standard plasmid (10 – 10^6 copies) or non-template control (0) are shown as dashed lines. Zymolyase-100T (1 unit) and Lyticase (10 units) are shown as solid lines. Standard curves were derived from copy numbers and C_T of the plasmid standards (open circles). Standard deviation of 8 measurements per sample were smaller than the symbols.

formed in a Class 100 clean work station equipped with HEPA filters and UV lamp (Airtech Japan Co., Ltd., Tokyo, Japan). Aerosol resistant tips (Molecular Bioproducts, Inc., San Diego, CA), PCR grade tubes (Axygen, Inc., Union City, CA) and sterile medical gloves (Nipro, K.K., Osaka, Japan) were used in every experimental step for prevention of airborne or other fungal contaminations. Primers and probes were synthesized by Applied Biosystems, Inc. (Foster City, CA). Sequences corresponding to the forward and reverse primers (NL-1¹⁶) and 260R¹³: primers for broad-range detection of fungal 28S rDNA) and the TaqMan probe were described elsewhere¹³, except the 5' reporter dye and 3' quencher which were modified to 6-FAM (6'-carboxyfluorescein) and NFQ-MGB (non-fluorescent quencher, and minor groove binder), respectively. MGB stabilized the probe interactions with the DNA template¹⁷, leading to enhancement of the lower limits of sensitivity of 100% detection rate of 28S rDNA to 6 copies per reaction from the original experimental method described by Vollmer *et al.*¹³ (data not shown). Stock solutions of freshly purchased Zymolyase-100T (Seikagaku Biobusiness, Tokyo, Japan) and Lyticase (L2524; Sigma-Aldrich, St. Louis, MO) were dissolved in UltraPure DNase/RNase-Free Distilled Water (Invitrogen) to 0.1–10 units/ μ l. Real-time PCR was performed in a total volume of 20 μ l consisting of 10 μ l of Premix Ex Taq (Perfect Real Time; Takara Bio, Shiga, Japan), NL1 and 260R primers at 0.2 μ M each, 15 mM TaqMan probe, 0.4 μ l

of ROX Reference Dye II (Takara Bio) in 96-well MicroAmp reaction plates (Applied Biosystems) with 1 μ l of diluted β -glucanase, or standard plasmids. Quantitative PCR monitoring was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems) with initial denaturation of 95°C for 30 s, followed by 60 amplification cycles of 95°C for 10 s and 60°C for 30 s.

RESULTS

The amount of dsDNA included in Zymolyase-100T and Lyticase preparations dissolved in water was estimated to be 304 pg and 40.4 pg per unit, respectively. Zymolyase included approximately 7.5 times more DNA than Lyticase. The amplification curves of each plasmid standard, 1 unit of Zymolyase, and 10 units of Lyticase are shown in the Fig. 1. Under the present experimental conditions, Lyticase at 1 unit per reaction was undetectable. Mean C_T of the plasmids at given copy numbers and the standard curve are also shown in the figure. From the standard curve, the average copy numbers of large subunit ribosomal DNA in these enzymes were calculated to be 9210 copies/unit for Zymolyase-100T and 0.0323 copies/unit for Lyticase. Different lots of these enzymes were tested (two or three lots each), and the distributions of DNA contaminants was similar (data not shown).

DISCUSSION

Two groups investigated DNA contamination using

PCR and agarose gel electrophoresis in 1999 using Zymolyase-20T supplied by Seikagaku Biobusiness and ICN Biomedicals^{11,12)} (both packaged enzymes derived from Kirin Brewery). The amounts of DNA detected in these studies were rough indications, as the positive and negative results were correlated with the total PCR cycle numbers, experimental conditions, and sensitivity of agarose gel electrophoresis detection with dye staining. In the present study, we have revealed the specific amounts of DNA contamination for further consideration of the effects of β -glucanases used in molecular diagnostics or genetic studies of fungi.

There were sufficient amounts of fungal DNA in Zymolyase-20T to interfere with panfungal fluorescent probe PCR (data not shown). We further analyzed Zymolyase-100T produced with additional affinity chromatography instead of Zymolyase-20T examined in two other studies, with the expectation of increased purity and removal of contaminants. However, Zymolyase-100T still contained an excess of fungal DNA for modern PCR applications. The amount of DNA included in these two β -glucanases differed by 290,000 times per unit. For example, 1 unit of Zymolyase-100T contained DNA equivalent to 46 – 92 yeast cells^{18, 19)}. On the other hand, Lyticase contained a much smaller fraction of DNA with realistic enzyme usages in most biological applications and molecular diagnoses (approx. 1 rDNA copy for 30 units of Lyticase). This result may have been due to the different methods used for purification of Lyticase^{10, 20)}, particularly related to the early carboxymethyl cellulose column step that can fractionate DNA by electrostatic interactions.

More than a decade ago, some groups warned that many companies were not aware that their products might be used for fungal PCR. Here, we revealed the actual amount of contaminating DNA in Zymolyase and Lyticase preparations. PCR applications for fungal diseases, such as deep mycoses including aspergillosis and candidiasis, and PCR-based environmental fungal detection methods are increasing. Nevertheless, we have realized that many of the scientists studying fungi have not been well advised about these DNA contaminations, except for partial members of society in mycology (personal communications). We are transmitting our results to the manufacturers of these products with the hope of improving their applicability to sensitive diagnosis methods. Until then, cautious decisions are required in the selection of products by those who prepare DNA with β -glucanase.

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References

- 1) Reiss E, Tanaka K, Bruker G, Chazalet V, Coleman D, Debeaupuis JP, Hanazawa R, Latgé JP, Lortholary J, Makimura K, Morrison CJ, Murayama SY, Naoe S, Paris S, Sarfati J, Shibuya K, Sullivan D, Uchida K, Yamaguchi H: Molecular diagnosis and epidemiology of fungal infections. *Med Mycol* **36**: 249-257, 1998.
- 2) Balajee SA, Sigler L, Brandt ME: DNA and the classical way: identification of medically important molds in the 21st century. *Med Mycol* **45**: 475-490, 2007.
- 3) Millar BC, Xu J, Moore JE: Risk assessment models and contamination management: implications for broad-range ribosomal DNA PCR as a diagnostic tool in medical bacteriology. *J Clin Microbiol* **40**: 1575-1580, 2002.
- 4) Mifflin TE: Chapter 1: Setting up a PCR laboratory. *In* PCR Primer: A Laboratory Manual, Second Edition (Dieffenbach CW and Dveksler GS ed), pp.5-14, Cold Spring Harbor Laboratory Press, New York, 2003.
- 5) Fredricks DN, Smith C, Meier A: Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR. *J Clin Microbiol* **43**: 5122-5128, 2005.
- 6) Griffiths LJ, Anyim M, Doffman SR, Wilks M, Millar MR, Agrawal SG: Comparison of DNA extraction methods for *Aspergillus fumigatus* using real-time PCR. *J Med Microbiol* **55**: 1187-1191, 2006.
- 7) Ferrer P: Revisiting the *Cellulosimicrobium cellulans* yeast-lytic β -1,3-glucanases toolbox: A review. *Microb Cell Fact* **5**: 10, 2006.
- 8) Beshay U, Miksch G, Friehs K, Flaschel E: Integrated bioprocess for the production and purification of recombinant proteins by affinity chromatography in *Escherichia coli*. *Bioprocess Biosyst Eng* **32**: 149-158, 2008.
- 9) Kitamura K, Yamamoto Y: Purification and properties of an enzyme, zymolyase, which lyses viable yeast cells. *Arch Biochem Biophys* **153**: 403-406, 1972.
- 10) Scott JH, Schekman R: Lyticase: endoglucanase and protease activities that act together in yeast cell lysis. *J Bacteriol* **142**: 414-423, 1980.
- 11) Loeffler J, Hebart H, Bialek R, Hagmeyer L, Schmidt D, Serey FP, Hartmann M, Eucker J, Einsele H: Contaminations occurring in fungal PCR assays. *J Clin Microbiol* **37**: 1200-1202, 1999.
- 12) Rimek D, Garg AP, Haas WH, Kappe R: Identification of contaminating fungal DNA sequences in Zymolyase. *J Clin Microbiol* **37**: 830-831, 1999.
- 13) Vollmer T, Störmer M, Kleesiek K, Dreier J: Evaluation of novel broad-range real-time PCR assay for rapid detection of human pathogenic fungi in various clinical specimens. *J Clin Microbiol* **46**: 1919-1926, 2008.
- 14) Makimura K, Mochizuki T, Hasegawa A, Uchida K, Saito H, Yamaguchi H: Phylogenetic classification of

- Trichophyton mentagrophytes* complex strains based on DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. J Clin Microbiol **36**: 2629-2633, 1998.
- 15) Sugita C, Makimura K, Murakami A, Murai Y, Yamaguchi H, Nagai A: A case of pulmonary aspergilloma molecular biological identification and typing of the isolates from antemortem sputa and autopsy fungus ball. Mycoses **46**: 149-152, 2008.
- 16) Kurtzman CP, Robnett CJ: Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. J Clin Microbiol **35**: 1216-1223, 1997.
- 17) Kutyavin IV, Afonina IA, Mills A, Gorn VV, Lukhtanov EA, Belousov ES, Singer MJ, Walburger DK, Lokhov SG, Gall AA, Dempcy R, Reed MW, Meyer RB, Hedgpeth J: 3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res **28**: 655-661, 2000.
- 18) Linkens MHK, Huberman JA: Organization of replication of ribosomal DNA in *Saccharomyces cerevisiae*. Mol Cell Biol **8**: 4927-4935, 1988.
- 19) Dammann R, Lucchini R, Koller T, Sogo JM: Chromatin structures and transcription of rDNA in yeast *Saccharomyces cerevisiae*. Nucleic Acids Res **21**: 2331-2338, 1993.
- 20) Kitamura K: Re-examination of Zymolyase purification. Agric Biol Chem **46**: 2093-2099, 1982.