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Journal of Food and Drug Analysis, Vol. 13, No. 2, 2005, Pages 184-191 藥物食品分析 第十三卷 第二期

Fluorescence versus Radioactivity for Assaying Antifungal Compound Inhibited Yeast 1,3-β-glucan Synthase Activity

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(Received: December 3, 2004; Accepted: March 15, 2005)

ABSTRACT

Yeast membrane (1,3)-β-glucan synthase (GS; EC 2.4.1.34) activity was assayed by a fluorescent dye-binding method and a conventional radioactivity method to compare its *in vitro* inhibition by the echinocandin-class antifungal compound. In the fluorescence method, GS-containing microsomal plasma membrane was first reacted with UDP-glucose substrate to form the 1,3-β-glucan product. The product was then solubilized and bound specifically with aniline blue to form a 1,3-β-glucan-fluorochrome dye complex for reporting the GS activity. The effect of each component in the GS assay buffer on fluorescence was examined. A calibration curve was constructed using yeast glucan as standard. In addition, the GS activities using the same amount of 1 μg/μL microsomal concentration in the two assays were compared. The radioactivity assay showed 38.3 μM UDP-glucose incorporation and corresponded to the formation of equivalent 262 μM yeast glucan in the fluorescent assay. It indicated a possible underestimation of 1,3-β-glucan products in the radioactivity assay or an overestimation of the products in the fluorescence assay. However, pneumocandin A_0 exhibited an IC_{50} value of 1.25 μM in the fluorescent assay closely comparable with that of 1 μM in the radioactivity assay. In summary, both the fluorescence and radioactivity tests generated similar data and pneumocandin inhibited GS. Possible limitations in the radioactivity assay were also discussed.

Key words: 1,3-β-glucan synthase, aniline blue, antifungal, yeast, echinocandin

INTRODUCTION

The 1,3-β-glucan synthase (GS; EC 2.4.1.34) located in the plasma membrane not only catalyzes the biosynthesis of (1,3)-β-glucan, which is an essential structural polymer of fungal cell wall, but is also a potential antifungal target against pathogenic yeasts[(1)]. Drugs used in chemotherapy are designed to distinguish plant and animal hosts from fungal pathogens. The ideal antifungal drug is one which has no adverse effects on the host but has a broad spectrum of action. Current therapeutic antifungal agents include amphotericin B, a polyene antibiotic that binds to the sterols of cell membranes and leads to cellular leakage[(2)], 5-fluorocytosine which interferes with DNA and RNA synthesis[(3)], or azole compounds which inhibit cytochrome p-450 enzyme in fungal endoplasmic reticulum and lead to the inhibition of ergosterol synthesis[(4)]. Although all of the above drugs are being used clinically, they may introduce toxicity to host cells and cause side effects. This fact results in the selection of GS as an alternative drug target. Preclinical antifungal drugs which inhibit specific fungal targets and yet cause minimal toxicity to the host have been intensely investigated for decades. Antifungals against GS originate from microbial sources and are structurally classified into three groups. One is the cyclic lipopeptide echinocandin B (ECB)-like inhibitors such as aculeacin A[(5)], cilofungin[(6)], pneumocandins[(7)], and LY 303366[(8)]. Another group of GS-targeting antifungals is the cyclic glycolipids such as papulacandin B[(9)], L-687,781[(10)] and BU-4794F[(11)]. ECB-class GS inhibitors or their combination with conventional antifungal drugs have been clinically evaluated in treating immunocompromised individuals such as HIV patients who suffer from opportunitistic fungal infections[(12)]. The third class of GS inhibitor was the recently found acidic terpenoid[(13)].

Usually GS activity is assayed in a radioactive system[(14)], GS activity is calculated by the incorporation of isotope-labeled UDP-[^{14}C]-glucose substrate into 1,3-β-glucan polymers, which are trapped on filter discs after separating unreacted substrate by serial washing. GS inhibition assay for the *in vitro* antifungal activity of tested compounds thus measures the reduced levels of substrate incorporation in the reaction mixture. However, the radioactive UDP-[^{14}C]Glc is expensive, and the washing waste is not environmentally safe. These concerns led to our objective of using the fluorescence-based, non-radioisotopic assay as a substitute in measuring GS activity and its inhibition in the research laboratory.

Aniline blue, a known fluorochromal triarylmethane, has long acted as a fluorescent probe in botanical histochemistry or structural topology studies for staining the wound-induced sugar, “callose”, on the plant’s new cell wall[(15-17)]. It is also applied in staining the septum (budding scar) of the live fission yeast *Schizosaccha-*

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romyces pombe for cell cycle kinetic studies[18]. Recent report showed its use in labeling cell wall 1,3-β-glucans for characterizing *Pneumocystis carinni* which is a major cause of opportunistic infection in AIDS patients[19]. The active fluorophore 4,4'-[carbonyl-bis(benzene-4,1-diyl)-bis-(imino)]-bis-benzenesulfonic acid in the aniline blue dye complex mixture is identified by mass spectroscopy, designated sirofluor. It is the major component in the dye which can form a specific complex with 1,3-β-glucans[20]. Much attention is given to the dye in the structural-functional activity relationship study of 1,3-β-glucans, known as biological response modifiers[21]. Besides being used in the above mentioned antifungal drug research, aniline blue has also been used in the development of 96-well microtiter assay for GS activity in the search for specific inhibitors[22]. The fluorescence emitted from aniline blue-1,3-β-glucan complex in the absence or the presence of increasing amount of GS-targeted inhibitors in the defined GS assay is measured by fluorescence spectroscopy. It shows that the microtiter assay is sensitive and is able to reduce the cost of each GS assay in higher than 100-folds compared to the conventional radioactivity assay. Therefore, we adopted the fluorescence method for GS activity assay. In this study, however, we adjusted the method slightly by maintaining the same condition of reaction space and time within the microfuge tube size for GS reaction as in the conventional radioactivity method in order to compare the two methods. Furthermore, glucan calibration curves constructed from the fluorescence of the yeast glucan standard bound to the aniline blue dye were used to extrapolate the concentration of glucan formed by GS in the fluorescence method. We also compared the quantities of glucan formation from the fluorescence and the conventional radioactivity method that directly reported the molar incorporation of ^{14}C-labeled substrate UDP-Glc of the recovered product. Loss of water soluble glucan signals from the radioactivity method was found and evaluated. The IC_{50} values obtained by the two methods for the antifungal *in vitro* activity of pneumocandin A_0, an ECB-class lipocyclopeptide which inhibits the microsomal GS activity of *C. albicans*, were also compared.

MATERIALS AND METHODS

I. *Materials*

Bakers yeast *Saccharomyces cerevisiae* (ATCC2366) and a pathogenic yeast *Candida albicans* (ATCC10231) that widely used to assay for antifungal antibiotics, were purchased from the Bioresource Collection and Research Center (BCRC) of the Food Industry Research & Development Institute (Hsinchu, Taiwan). Radioactive [^{14}C] uridine diphosphate glucose (UDP-[^{14}C]Glc; 287.4 mCi/mmol) and liquid scintillation supplies were obtained from NEN Life Science (Boston, MA, USA). Brij 35 (polyoxyethylene ether, 23 lauryl ether) was purchased from Pierce (Rockford, Il USA). Guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) was obtained from Boehringer Mannheim (Indianapolis, IN, USA). Pneumocandin A_0 was a gift from Merck Sharp & Dohme Corp. (Rahway, NJ, USA). Growth media were obtained from Difco (Detroit, MI, USA). The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Richmond, CA, USA). Glass beads were purchased from BioSpec (Bartlesville, OK, USA). BSA (fatty acid-free), β-lactoglobulin (β-LG), aniline blue (Sigma M5528, CI 42780), yeast glucan (Sigma G5011) and all the other chemicals were purchased from Sigma Chemical Co (St. Louis, MO, USA).

II. *Growth Conditions*

S. cerevisiae and *C. albicans* were maintained on YEPD (1% yeast extract, 2% peptone, 2% dextrose) medium plates containing 2% (w/v) agar. Cells were first grown at 30°C in YEPD medium for 12 hr at 200 rpm, and then diluted 100 times into fresh YEPD medium to grow 4 hr to mid-log phase. They were harvested by centrifugation at 3,000 ×g for 10 min at 4°C and then suspended and washed with cold TE buffer (70 mM Tris-HCl, 3 mM EDTA, pH 7.0). The suspended cells were centrifuged at 3,000 ×g for 10 min at 4°C again to recover the cell pellets and weighed.

III. *Microsomal Membrane Preparation*

Microsomal membranes were prepared by differential centrifugation. All steps were performed on ice and centrifugation was set at 4°C. Wet cell paste was first suspended in cold homogenizing buffer containing 25 mM sucrose, 3 mM EDTA, 25 μM GTP, 25 mM β-mercaptoethanol, 3 mM EGTA, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 70 mM Tris-HCl (pH 7.5) at 5 g/mL ratio. Then cells were mixed with prechilled 0.5 mm glass beads at 16 g/25 mL ratio and homogenized by a Bead Beater (Biospec products Bartlesville, OK, USA). The beater was installed inside a cold jacket filled with ice water. Cell solution was operated with five 20s bursts at 5 min cooling intervals.

The homogenate was centrifuged for 10 min at 1,500 ×g to remove cell debris. The supernatant was centrifuged again at 23,000 ×g for 10 min to remove mitochondrial membranes into pellet. The supernatant was subjected to ultracentrifugation for 1 hr at 100,000 ×g to recover microsomal membranes in the final pellet. The membrane pellet was well suspended in cold storage buffer (1 mM EDTA, 1 mM DTT, 33%(v/v) glycerol and 50 mM Tris-HCl, pH 7.5) by passage through gauge 18, 23, and 26 needles successively. The resulting membrane vesicle concentrations were adjusted to 10~20 mg/mL, and then stored in aliquots at -80°C. Membrane vesicles were thawed on ice before use. Protein concentration was determined by coomassie brilliant blue dye-binding method[23] using BSA as standard.

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IV. *GS Activity Assay by Fluorescence Method*

The fluorescence assay for GS activity consisted of two stages. The first stage was GS reaction in which for *S. cerevisiae*, microsomal membranes (0 - 4.5 μg/μL) were suspended in 0.1 mM GTP, 1 mM EDTA, 1.4% (v/v) glycerol, 0.8 mg/mL BSA, (for component effect, BSA or β-LG at 0.08-8 mg/mL were used), 75 mM Tris-HCl (pH 8.0) and 1 mM UDP-Glc in a total volume of 100 μL to react at 30˚C for 30 min. For *C. albicans*, assays were conducted in a mixture of 100 μL containing final concentrations of 20 μM GTP-γ-S, 1 mM EDTA, 8% (v/v) glycerol, 0.5% (v/v) Brij-35, 80 mM Tris-HCl (pH 7.8), 0-1.5 μg/μL microsomes and 2 mM UDP-Glc. The GS reaction was terminated by an addition of 200 μL of 1.5 N NaOH. The second stage was the aniline blue dye-binding stage in which the above 300 μL sample was added with 30 μL of 6 N NaOH and then immediately incubated at 80˚C for 30 min to solubilize the newly formed glucan. Then 630 μL of dye solution (ratio for 0.1% aniline blue in water, 1 N HCl, and 1 M glycine in NaOH buffer, pH 9.5, was 40/21/59, with a final dye mix pH of 3.4) was added to react at 50˚C for 30 min to form glucan-dye fluorochrome complex. The unbound fluorescent dye was quenched at room temperature for 30 min while fluorescence of the bound form was measured with a fluorescence spectrophotometer (HITACHI, F-2000, Japan) at excitation wavelength of 400 nm and emission wavelength of 460 nm.

To construct glucan calibration curves, yeast glucan standard at various concentrations was dissolved in 300 μL of 1 N NaOH, followed by the second stage procedure. Each solution was prepared at its defined concentration as in the GS assay buffer in a total volume of 300-μL 1N NaOH, followed by the second stage procedure to measure its fluorescence. Effect of each component in the above mentioned GS assay buffer on fluorescence signal was also tested. All samples were performed in duplicates.

V. *GS Activity Assay by Radioactivity Method*

For *S. cerevisiae*, regular assays were conducted in a mixture of 100 μL containing final concentrations of 0.1 mM GTP-γ-S, 1 mM EDTA, 1.4% (v/v) glycerol, 0.8 mg/mL BSA, 75 mM Tris-HCl (pH 8.0), 0 - 1.5 μg/μL microsomes and 1 mM UDP-[^{14}C]Glc (0.12 mCi /mmol). For *C. albicans*, assays were conducted in a mixture of 100 μL containing final concentrations of 20 μM GTP-γ-S, 1 mM EDTA, 8% (v/v) glycerol, 0.5% (v/v) Brij-35, 80 mM Tris-HCl (pH 7.75), 1 μg/μL microsomes and 2 mM UDP-[14C]Glc (012 mCi/mmol). Reaction mixture was incubated at 30˚C for 30 min and terminated by spotting the whole mixture onto a Whatman GF/A 2.4 cm filter disc on a heating block set at 120˚C to dry for 10 min. Then, the filter discs were washed successively with 66% (v/v) ethanol containing 85 μM EDTA, 66% ethanol, and 70% ethanol[24] in a filtration manifold apparatus (Millipore, Bedford, Mass, USA). Each filter disc which contained the trapped ethanol-insoluble [^{14}C]-glucan product polymer was added with 5 mL of scintillation cocktail and measured by a scintillation counter (Beckman LS-6500, Beckman Coulter, Inc. Fullerton, CA). All samples were conducted in duplicates.

To measure the loss of glucan products during the washing step in the radioactivity assay, the experiment was performed similarly as described above except that only unlabelled UDP-Glc substrate was used. In addition, during the washing step, the filtrate fractions from the manifold apparatus were collected separately in test tubes inside the apparatus chamber. The filtrate was subjected to centrifugation at 13,000 ×g for 10 min at 4˚C to recover the glucan pellet. The pellet was dissolved and diluted in 300 μL of 1 N NaOH. Then, the glucans were resolubilized by adding another 30 μL of 6 N NaOH and incubated at 80˚C for 30 min before aniline blue dye solution was added as described in the second stage of the fluorescence assay.

RESULTS

Before beginning of experiments, we optimized the fluorescence measuring parameters for consistent fluorescent intensity and sensitivity with low background noise from band path width and output voltage levels. The slit width of 20 nm and output voltage of 700 for both excitation and emission conditions were determined and then used throughout the study.

I. *Construction of Glucan Fluorescence Calibration Curve*

Commercial yeast glucan was used as standard to mimic the GS product synthesized in the yeast GS reaction to construct the glucan fluorescence calibration curve. The calibration curves for both broad and narrow ranges were established. Various concentrations of yeast glucan were prepared based on its dehydroxyl glucose unit, monomer molecular mass of 162 gm, as one mole for calculation. Figure 1 exhibits a linear correlation between yeast glucan concentrations within a 2000 μM broad range and the fluorescence emitted from the aniline blue-bound glucan complex. The linearity was also obtained within a 100 μM glucan concentration narrow range as drawn in the inlet figure. In this study, the regression formula in the broad range calibration curve was used to extrapolate the fluorescence intensity in the GS fluorescent activity assay into their corresponding 1,3-β-glucan production when the net fluorescence intensity in the sample was higher than 50. If the net fluorescence intensity value of the sample was less than 50, the narrow range calibration curve was used. In other words, these two plots were used to convert the fluorescence signal into 1,3-β-glucan concentration formed in the GS assay in order to compare the values of 1,3-β-glucan incorporated by the radioactivity assay.

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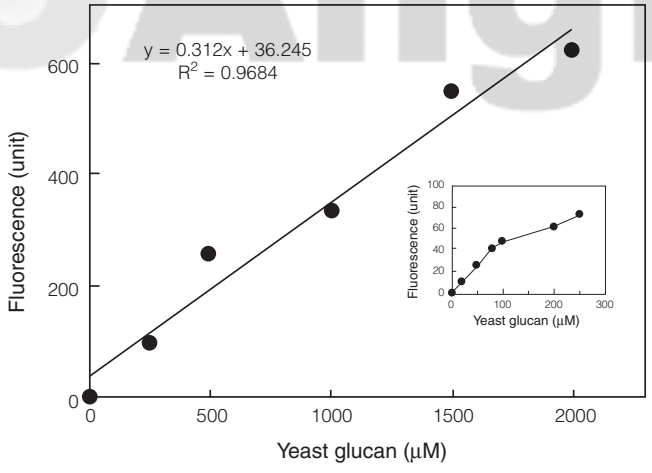

Figure 1. The glucan fluorescence calibration curve.
The inlet figure is the standard curve of small concentration range. The stock 2000 μM yeast glucan was first prepared in 1N NaOH solution. It was then dissolved at 80°C for 30 min before preparing various concentrations in 1N NaOH with final sample volumes of 300 μL. Aniline blue binding with glucan was then conducted from the second stage as described in the material and methods section.

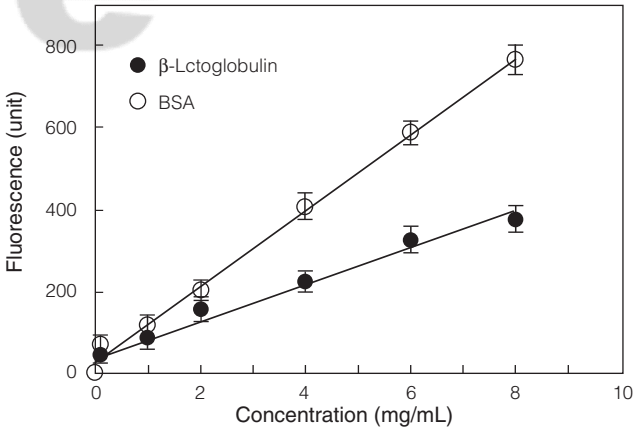

Figure 2. The effect of protein components on the fluorescence of aniline blue.
Various concentrations of BSA or β-lactoglobulin were first prepared in 100 μL of 75 mM Tris-HCl (pH 8.0) buffer and added with 200 μL of 1.5 N NaOH. Aniline blue binding was then conducted from the second stage as described in the material and methods section.

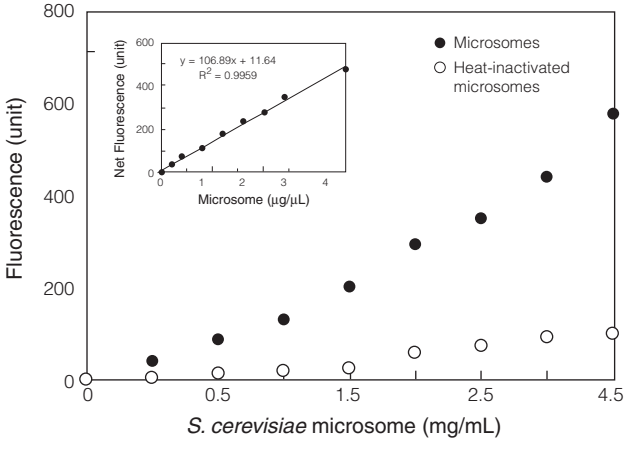

Figure 3. GS activity assay for yeast microsomes by the fluorescence method.
Yeast microsomes and the heat-inactivated forms were assayed in parallel for the dye-induced fluorescence of glucan products after GS reaction. The inlet figure is plotted from the net fluorescence of GS synthesized glucan, which was obtained by subtracting the fluorescence of glucan product of yeast microsomes from that of heat-inactivated forms after GS reaction. Heat-inactivated yeast microsome samples were prepared by heating a solution of 20 mg/mL microsomes at 100°C for 10 min before preparing the first stage 100 μL of GS reaction mixtures.

II. *The Effect of Assay Buffer Components on Fluorescence*

It is known that any substance containing aromatic groups or conjugated double bonds is a potential fluorophore which exhibits fluorescence when energy is excited high enough at a desirable wavelength[25]. Therefore, we tested whether there is interfering fluorescence from each individual component in the GS assay buffer for *S. cerevisiae* and *C. albicans* including BSA, β-LG, GTP, EDTA, glycerol, Brij-35, Tris-HCl, and UDP-Glc. It was assumed that they might disturb the fluorescence of aniline blue when their solution was excited at 400 nm and fluorescence emission was measured at 460 nm. Results showed that only components of protein nature (BSA and β-LG) for *S. cerevisiae* possessed fluorescence (Figure 2). BSA of 0.08 to 8 mg/mL concentrations exhibited fluorescence of 70 to 764 units, and an average of half the values were also found to be associated with β-LG, suggesting that their concentrations in the GS assay buffer were necessarily to be minimized. Therefore, in order to avoid the background fluorescence of these protein components, but maintain decent GS activities and reflect only the fluorescence from aniline blue-bound glucan products in the GS fluorescence activity assay, both BSA and β-LG in the GS assays were modified to 0.8 mg/mL. This concentration was amounted to 1/10 of their original uses for *S. cerevisiae* in both radioactive and fluorescent assays.

III. *GS Activity Assay for S. Cerevisiae by Fluorescence Method*

Similar to the above concerns, the existence of microsomal proteins, also possessing potential fluorescence from the aromatic amino acids, caused interference in the fluorescence GS assay as expected. Therefore, to make the fluorescence approach qualify for GS assay, this protein effect was corrected by deducting the fluorescence of heat-inactivated microsome samples from the same amount of active microsome counterparts after their GS reactions were conducted in parallel. In this experiment, increasing levels of yeast microsomes of the paired samples were performed (Figure 3). A linear correlation was again obtained between the net fluorescence from the glucan synthesized and the concentration of microsomes incubated in the GS reaction

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(Figure 3 inlet graph). It demonstrated that GS activity measured by the fluorescent assay was apparently in a dose-dependent manner. The net fluorescence value of the GS assay was used to calculate the amount of 1,3-β-glucan produced from the glucan calibration curves (Figure 1).

IV. *GS Activity Assay for S. cerevisiae by Radioactivity Method*

In the radioactivity method, GS activity was expressed as the unit time production of the moles of UDP-^{14}C-Glc incorporation into the alcohol-insoluble glucan product, which was trapped on filter discs after filtration and washing process. The GS substrate in this radioactive assay consisted of a mixture of isotope labeled UDP-^{14}C-Glc and unlabeled UDP-^{12}C-Glc (hot/cold mix). The specific radioactivity for the final concentration of 1 mM hot/cold UDP-^{14}C-Glc substrate used in 100 μL assay volume for *S. cerevisiae* was 0.12 mCi/mmole (2.64×10^8 cpm/mmole, counting efficiency = 1). Therefore, the total radiation of 26,400 cpm amounted to the given total 100 nmole of UDP-Glc substrate molecules. According to the radiation counting of the trapped product, the moles of UDP-Glc incorporation were calculated from the ratio of the radiation of the product to the given 26,400 cpm, which was then multiplied by 100 nmole of UDP-Glc. The UDP-Glc conversion in molarities in the reaction solution was then derived as noted in Figure 4. Figure 4 shows that there was linear correlation of microsome concentration either with product radiation or with incorporated UDP-Glc concentration, indicating that GS activity measured by the radioactive assay was apparently also in a dose-dependent manner (Figure 4).

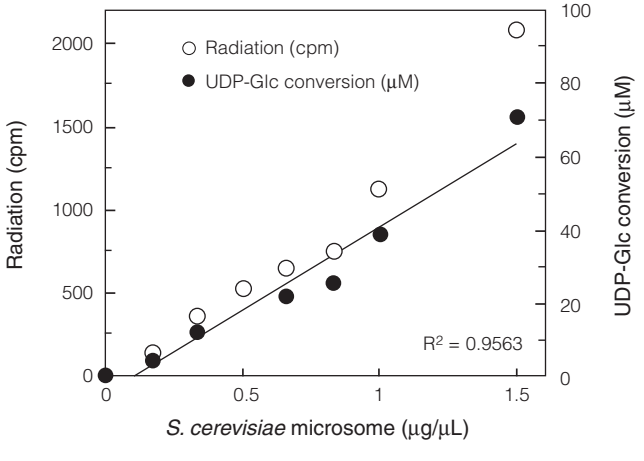

Figure 4. GS activity assay for yeast microsomes by the radioactivity method.
The left ordinate shows the radiation counting (cpm) from the glucan product. The right ordinate shows the corresponding concentration of converted substrate UDP-glucose (μM) into the glucan polymers. Concentration of UDP-Glc conversion (μM) = Net radiation (cpm)/ total 26,400 (cpm) × 100 nmole/0.1 mL. Net radiation was obtained from the difference between yeast microsomes and heat-inactivated microsomes after GS reaction.

Owing to the reasoning that 1,3-β-glucan product is the reporter for GS activity, the concentrations of glucan formation in both methods were compared based on assaying the same enzyme source of microsomes at 1 μg/μL. In the fluorescence method, Figure 1 and Figure 3 were integrated in which the corresponding net fluorescence value of GS product formed by 1 μg/μL yeast microsome (~118 units in Figure 3 inlet) was used to calculate the concentration of yeast glucan formation from the regression formula in Figure 1. 38.3 μM UDP-Glc incorporation (Figure 4) was detected with the radioactivity method, and it corresponded to the detection of equivalent 262 μM yeast glucan formation with the fluorescence method. Thus, it seemed that there was a seven-fold difference of the reported signals between the two methods.

V. *Evidence of Undetected 1,3-β-glucan Product in the Filtrate of the Radioactive Assay*

The seven-fold difference possibly implied an underestimation of 1,3-β-glucan product in the radioactive assay. We thus examined this possibility by collecting the filtrate fractions from a modified radioactive assay for *C. albicans* microsomes, followed by fluorescent assay to detect whether any 1,3-β-glucans products might be washed into the filtrate and escaped from the filter discs for counting. We found the filtrate collection from the unlabeled GS assay of *C. albicans* indeed contained 1,3-β-glucan

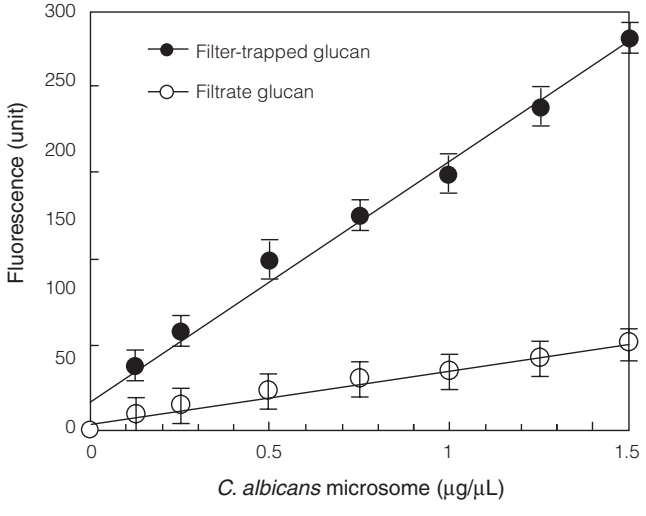

Figure 5. Glucan was found in the filtrate fraction during washing of the GS assay products for *C. albicans*.
Various concentrations of GS-containing *C. albicans* microsomes were used in the first stage GS assays in the two sample sets. After GS reaction, one set of the reaction mixtures was put directly through the second stage aniline blue binding experiment to record the fluorescence from total glucan products (the total glucan line). Another set of the reaction mixtures was spotted onto the filter discs and washed successively with 66% (v/v) ethanol containing 85 μM EDTA, 66% ethanol, and 70% ethanol as described in the radioactive assay, in order to collect the filtrate fractions. The filtrate fractions were then subjected to assaying glucan content by the fluorescent assay (the filtrate glucan line).

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products (Figure 5). And there was actually up to 20% loss of glucan during the serial washing steps.

VI. *Comparison of Antifungal Activity of Pneumocandin A_o in the Fluorescent and Radioactive Assays*

We used pneumocandin A_0[26] as a positive control compound to compare its antifungal activities against pathogenic *C. albicans* GS in the two methods. Its fluorospectral feature was similar to that of pneumocandin B_0, which exhibited an excitation maximum at 275 nm and an emission maximum at 303 nm[27] and had no interference with the fluorescent reporter, aniline blue, which measured at Ex 400 nm/Em 460 nm. Besides, there was no nonspecific fluorescence quenching to the emission of aniline blue-bound glucan complex by pneumocandin A_0 when it was added into the GS assay mixture. Figure 6 shows that pneumocandin A_0 had IC_{50} value of 1 μM from the radioactivity method and 1.25 μM from the fluorescence method.

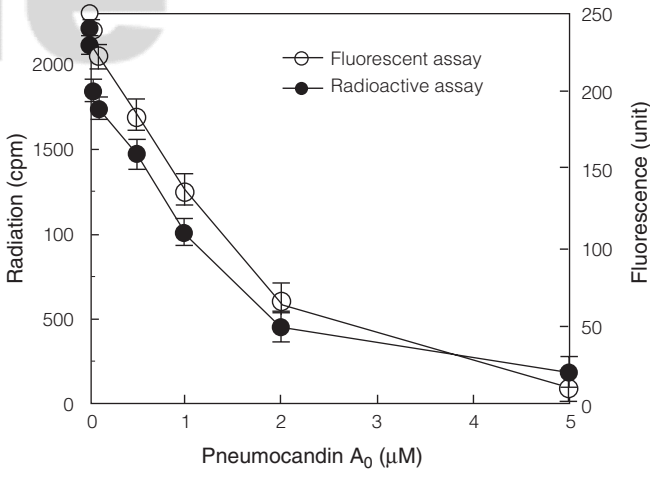

Figure 6. A comparison of the inhibition of GS activity of *C. albicans* microsomes by pneumocandin A0 between the radiation and fluorescence assays.
Pneumocandin A0 was prepared in DMSO of various concentrations in 10 μL. It was incubated with GS reaction mixture for 10 min on ice before UDP-Glc was added for both assays. The DMSO at 10 μL in the GS assay mixture exhibited no influence on the fluorescence of aniline blue.

DISCUSSION

This study reports a modified version of the microtiter-based method[22] to detect GS activities for antifungal assay in a common research laboratory, where the microplate fluorescence reader is not usually available. We relied on the principle of fluorescence detection as a good approach to resolving the safety problem of the waste caused by the conventional radioactivity method. However, by comparing the formation of 1,3-β-glucan product with the same amount of yeast microsomes at 1 μg/μL, we calculated a seven-fold difference in the two GS assay systems. This outcome difference might be raised by either an overestimation from the fluorescence method or underestimation from the radioactivity method. We propose several possible reasons as follows. The first is the non-homogeneity of the commercial yeast glucan products; some of which might have been contaminated with residual yeast cell wall components such as mannan and chitin, resulting in overcounting of the real number of glucan molecules in the sampling weight during the construction of glucan fluorescence standard curve. Therefore, the fluorescence value was contributed by the 1,3-β-glucan portion from a given amount of heterogeneous yeast glucan products. This factor was able to cause overestimation of the glucan product when we did the fluorescence conversion with the standard curve in Figure 1. Yeast mannan in particular exhibited 1/52 of the aniline blue-induced fluorescence upon binding as compared to yeast glucan examined previously[20]. This factor may also cause minor miscalculation of glucan product concentration from the corresponding fluorescence value in Figure 1.

Secondly, the aniline blue has bound with the newly synthesized glucan products much stronger than with the standard yeast glucan, resulting in higher fluorescence signals that were detected within the new GS products. Because the association of the aniline blue dye with 1,3-β-glucan is dependent on its stereo-chemical conformation in solution state[28], the fresh product may form a better helical structure for the dye to access easier and thus have higher binding stoichiometry than with the yeast glucan standard. Moreover, it was reported that the fluorescence induced by the dye mixture might not indicate the presence of 1,3-β-glucan due to the impurities in the aniline blue chemical, which exhibited fluorescence upon binding with endogenous sugars other than 1,3-β-glucan in the glucan standard. In addition, the fluorescence might cause overestimation as well[29].

In addition to the factors discussed above, the main variable to product quantification by the fluorescence method was the selection of the glucan standard[22]. The 1,3-β-glucan species of single helical conformation preferentially reacts with aniline blue in solution was reported[28]. If the binding behavior of a new 1,3-β-glucan standard with the aniline blue dye changes will change the slope of the new glucan calibration curve and change the ultimate glucan quantification accordingly. Since enzyme specific activity (mol $min^{-1}mg^{-1}$) is proportional to the quantity of product formation, the application of the fluorescence assay in enzyme kinetic study would probably not be popular until the discovery of a homogenous, acceptable and well-recognized source as the 1,3-β-glucan standard just as BSA has been used as a standard for protein quantification by the principle of protein-dye binding in the Bradford method for decades[23]. We should not, however, rule out the possibility of using the fluorescence method as a relative quantification for 1,3-β-glucans and enzyme kinetics if the standard is a well defined 1,3-β-glucan species.

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The fluorescence assay system has affected the detection sensitivity of the susceptibility of membrane GS to pneumocandin A_0. The 25% higher IC_{50} value from the fluorescence method than from the radiation method might be a reflection of the underestimation of synthesized glucan in the radioactive assay as described above. However, the difference was not as much as the seven-fold of the calculation from 1,3-β-glucan products, which was proportional to the specific activity of GS. The much less difference should be probably due to the fact that IC_{50} value is 50% inhibition of the enzyme activity as a whole instead of 50% of specific GS enzyme activity.

The action of ECB-class drug on GS was believed to be noncompetitive inhibition[30]. A few reports have demonstrated that the mechanism of GS inhibitors was the combined effects of direct and indirect actions. For example, the indirect inhibition on GS, by phospholipase A_2 and its reaction products, was due to the perturbation of lipophilic fatty acid side chains of the inhibitors on the membrane environment and the alteration of the membrane fluidity[24]. The direct visualization of the interaction of acyl side chains of ECB-class compound with membrane environment was demonstrated by using the drug as an inherent fluorescence probe in the fluorescence titration and steady-state emission anisotropy study[27]. A 40 kDa protein, further identified by photoaffinity labeling, was found to involve in the direct interaction of LY303366 with GS-containing membranes[31]. Therefore, the fluorescence method in this report should be applicable in studying the interaction of the ECB-class antifungal drug with membrane GS in the future because the drug and aniline blue dye possess independent fluorescence spectroscopic features.

This report shows a logistic demonstration of linkage experiments between the quantities of the fluorescence from the GS reaction, from the standard 1,3-β-glucans, the membrane background fluorescence subtraction and the radiation of glucan product. It shows that the fluorescence method is a proper substitute for the radioactivity assay in the detection of GS inhibitors. It supports the use of the fluorescence method as a way to avoid the potential drawbacks of the environmental hazard caused by radiation waste. In conclusion, the fluorescence assay is useful both in the GS activity studies in which the amount of glucan formed is not a critical factor, and is useful in studying the antifungal activity of the yeast GS-targeted inhibitory compounds such as ECB-class drugs.

ACKNOWLEDGEMENTS

This research was supported mainly by the National Science Council, Taiwan, R.O.C. grant no NSC88-2825-B-C-039-001-B and partially by NSC88-2314-B-039-034, and was conducted at Department of Nutrition, China Medical University, Taichung, Taiwan, R.O.C.

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