Inhibitory effect of coated mannan against the adhesion of *Candida* biofilms to denture base resin

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The adherence of *Candida* on dentures is related to diseases such as denture stomatitis and aspiration pneumonia. Mannan is a major component of the *Candida* cell surface, and contributes to the cell adherence. A previous report indicated that the adherence of *C. albicans* to culture dishes was inhibited by the coating them with mannan. The purpose of this study was to examine the adhesion inhibitory effect of mannan coating on acrylic denture surfaces against *C. albicans* and *C. glabrata*. The amount of *Candida* attached on the acrylic surfaces coated with mannan was calibrated by culture methods. Mannan showed significant inhibitory effects on *Candida* adhesion in both the yeast and hyphal form in a concentration-dependent manner, and the durability of the inhibitory effect continued for three days. These results suggest that mannan coating on the denture base acrylic can prevent *Candida* adhesion on the denture.

Keywords: *Candida albicans*, *Candida glabrata*, Mannan, Denture base resin

INTRODUCTION

The surface of denture base acrylic resin is porous, and denture plaque easily adheres to the surface of dentures. The colonization of microorganisms on the denture base acrylic resin occurs rapidly, and *Candida* species adhere strongly to denture base materials¹.

The prevalence of denture wearing was 49% in subjects aged 15 years and over, and 96.9% of these subjects were elderly, according to a survey of dental diseases in Japan². It has been reported that the carriage rate of *Candida* species in the oral cavity is increased by denture wearing, and *Candida albicans* and *Candida glabrata* were often detected^{3–5}. The adhesion of *Candida* species to denture surfaces is an important pathogenic factor not only because it induces the degradation of the denture base material, but also because it can cause denture stomatitis. Furthermore, poor denture hygiene might increase the risk of aspiration pneumonia in the elderly⁶.

Denture stomatitis is a very common disorder affecting denture wearers, and it has been reported in 11–67% of complete denture wearers⁷. Antifungal therapy is effective for the acute treatment of inflammation associated with denture stomatitis. However, the effectiveness of antifungal treatment is limited long-term, and rapid recurrence of denture stomatitis can occur shortly after stopping treatment. The recurrence of this disorder seems to be due to continuous infection by residual *Candida* species on the denture surfaces^{6,8}. The surface hydrophobicity of the materials increases the ability of the hyphal forms of *C. albicans* to colonize the denture surfaces, and *in vitro* studies have shown that the hydrophilic coating materials can decrease the

adhesion of *C. albicans*, but not other *Candida* species, thereby decreasing their ability to colonize the denture material surface^{6,9}.

The outermost layer of the *Candida* cell wall is covered with hydrophilic polysaccharides, such as mannan or galactomannan¹⁰. These mannans on the fungal surface function as adhesins, which are involved not only in the adhesion to the host cell^{11,12} but also in the adsorption to plastic plates. On the other hand, when the plastic surfaces of culture dishes were coated with mannan, the adherence of *C. albicans* to the dishes was significantly inhibited^{13,14}.

The β -1, 2-linked manno oligosaccharide in mannan shows strong antigenicity and is associated with the induction of tumor necrosis factor alpha (TNF- α), a proinflammatory cytokine^{10,15}. Mannan also promotes wound healing by increasing the proliferation of fibroblasts and vascular endothelial cells, and by enhancing the production of collagen¹⁶. Recently, food and medical devices coated with mannan have been developed, and a variety of effects of mannan have been confirmed^{17–19}. Based on the findings of these clinical reports, mannan appears to be effective for preventing the development of local and systemic diseases, including denture stomatitis.

The purpose of this study was to examine whether adding a mannan coating on the denture base acrylic surfaces can inhibit the adhesion of *C. albicans* and *C. glabrata*, which are often identified from patients with denture stomatitis, and to evaluate the duration of this effect of mannan on the adhesion of *C. albicans*.

MATERIALS AND METHODS

Preparation of denture base acrylic resin specimens

Acryl fluid resin for clear type dentures (Palapress Vario, Kulzer, Germany) was polymerized according to the manufacturer's instructions, and was trimmed into 3×3×2 mm pieces for the REDOX indicator assay and 25×25×2 mm pieces for the adherence inhibitory assay. All samples were sterilized with 80% ethanol, and immersed in sterilized distilled water (DW).

Preparation of reagents and coatings

Purified mannan derived from *Saccharomyces cerevisiae* (Sigma Chemical Co., MO, USA) at concentrations of 0.1 mg/mL, 1 mg/mL, 10 mg/mL, and 10 mg/mL of mannose (Wako Pure Chemical, Osaka, Japan) were used in this study. All of the solutions were sterilized by filtration. Sterilized acrylic pieces were immersed in the above three concentrations of mannan solution (mannan group) or mannose solution (mannose group) overnight. The control group (DW) was immersed in DW overnight. The saliva group was immersed in DW overnight and put into filtered human saliva. After immersion, all of the samples except for the control, were placed into the filtered human saliva, and the acrylic pieces were kept in saliva overnight.

Candida strains and culture conditions

1. *Candida albicans*

Candida albicans ATCC18804 was cultivated in Tryptic Soy Broth supplemented with 5% dextrose (TSBD; Becton, Dickinson and Company, NJ, USA) medium at 37°C with shaking for preculture. Two different media were used for *C. albicans* cultivation. For the hyphal form of *C. albicans*, a 300 µL aliquot of the preculture was inoculated into 3 mL of RPMI1640 medium supplemented with 10% fetal bovine serum (FBS; GIBCO, Invitrogen Co., CA, USA). For the yeast form of *C. albicans*, a 300 µL aliquot of the preculture was inoculated into 3 mL of TSBD. Both were cultivated with the acrylic pieces for 72 h. The number of *C. albicans* cells was adjusted to approximately 1×10^6 to 1×10^7 CFU/mL in the precultures.

2. *Candida glabrata*

Candida glabrata ATCC90030 was cultivated in TSBD medium at 37°C with shaking for precultures. The number of *C. glabrata* cells was adjusted to approximately 1×10^7 to 1×10^8 CFU/mL. A 300 µL of aliquot of the preculture was inoculated into TSBD with the acrylic pieces for 72 h.

Adherence inhibitory assay of the yeast form of Candida cells using the washing out method

The washing out method was slightly modified to use JIS Z 2801 as the antibiotic. After immersion into each mannan solution (0.1 mg/mL, 1 mg/mL, 10 mg/mL) or DW (control) overnight, 100 µL aliquots of *C. albicans* or *C. glabrata* suspensions, adjusted to approximately 1×10^6 to 1×10^7 CFU/mL with TSBD, were dropped onto

acrylic pieces, and the plates were covered with sterilized polyethylene film (20×20 mm). These acrylic pieces were incubated at 30°C for 72 h. After incubation, the acrylic pieces were washed with distilled water to remove non-adherent *Candida* cells. The adherent *Candida* were washed out from each of the acrylic pieces with the Soya casein digest lecithin polysorbate (SCDLP; Wako Pure Chemical, Osaka, Japan) medium, and the cell count in the washout liquid from the SCDLP was measured by a CFU assay.

REDOX indicator assay

A 300 µL aliquot of *C. albicans* or *C. glabrata* suspension culture was added into each 15 mL tube containing five acrylic pieces, and 3 mL of RPMI 1640 medium was used for *C. albicans*, while TSBD medium was used for *C. glabrata*. These tubes were placed in a shaker incubator for three to seven days at 37°C. After incubation, the acrylic pieces were immersed and washed in distilled water three times to remove non-adherent cells. The washed pieces were placed on 96 well plates, and 150 µL of the RPMI 1640 medium supplemented with 10% FBS and a REDOX indicator (Alamar blue; Trek Diagnostic Systems, Cleveland, OH, USA) was added, and were incubated again. The *C. glabrata* group was supplemented with 50 µL of 20% glucose, in addition to the RPMI 1640 medium containing the REDOX indicator.

The amount of adherent *Candida* was estimated based on the fluorescence levels (EX: 530, EM: 590) by a Fluorescent Microplate Reader Cyto-Fluor 2 (Per Septive Biosystems, Farmingham, MA, USA). The durability of the mannan effect was examined every day for one week.

To confirm the adherence of *C. albicans* biofilms, the acrylic pieces from the control group and 10 mg/mL mannan group were observed with microscopy at 400× magnification after Gram staining.

Statistical analysis

The statistical analyses were performed among multiple groups using the Kruskal-Wallis test, and between two groups using a Mann-Whitney U-test (SPSS 14.0 J). Significant differences were considered to be indicated by values of $p < 0.05$.

RESULTS

Adherence inhibitory assay of the yeast form of C. albicans cells by the washing out method

The amounts of the adherent yeast form of *C. albicans* detected by the washing out method are shown in Fig. 1. The CFU of *C. albicans* yeast cells were significantly decreased in a mannan concentration-dependent manner ($p < 0.05$). There were significant differences between each of the mannan groups and the control, and among the mannan groups ($p < 0.05$).

Fig. 1 The amount of adhered yeast form of *C. albicans* detected by washing out method. There was significantly difference between two groups (Mann-Whitney U-test, $p < 0.05$).

Inhibition of the adherence of the hyphal form of *C. albicans* as indicated by the REDOX indicator assay
The amount of *C. albicans* that adhered to the acrylic surfaces after incubation for 30 h is shown in Fig. 2a. In the control group, the saliva group and the mannose group, a large amount of *C. albicans* biofilm was noted to be present on all acrylic pieces, but there was no significant difference among these groups. The fluorescence level of the *C. albicans* that adhered to the acrylic pieces treated with 0.1 mg/mL of mannan was 45% of the level in the control group, and was 12% on the pieces treated with 1 mg/mL mannan, and only 1% on the pieces treated with 10 mg/mL mannan. There were significant differences between each of the mannan groups and the control, the saliva group and the mannose group, except for between the saliva group and the 0.1 mg/mL of mannan group ($p < 0.05$).

A micrograph of the *C. albicans* biofilms after Gram staining is shown in Fig. 2b. In the control group, a larger amount of *C. albicans* biofilms was observed on the acrylic pieces compared to those treated with 10 mg/mL of mannan.

The durability of the effect of mannan on *C. albicans* as determined by the REDOX indicator assay

The durability of the effect of the mannan treatments is shown in Fig. 3. The level of fluorescence in the mannan groups was significantly decreased ($p < 0.05$) in a mannan concentration-dependent manner on the first and second days, but the level was not decreased compared to the control group on the third, fourth or fifth days. On the third day, the control, 0.1 mg/mL and 1 mg/mL of mannan samples were significantly different from the samples treated with 10 mg/mL of mannan ($p < 0.05$). On the fourth and fifth days, there were no significant differences among the test groups. In the control group, the amount of fluorescence was the same for one week.

Fig. 2 The amount of adhered hyphal form of *C. albicans* on resin surfaces.

a: Redox indicator assay of hyphal form of *C. albicans*

Statistical analyzes of fluorescence levels were performed among multigroups by Kruskal-Wallis, and between two groups of control and each test group was analyzed by Mann-Whitney U-test, significant differences were judged as $p < 0.05$.

b: Micrograph of *C. albicans* biofilms after gram staining

In control group, a large amount of *C. albicans* biofilms on the acryl pieces were observed compared with 10 mg/mL of the mannan group.

Adherence inhibitory assay of the yeast form of *C. glabrata* cells by the washing out method

The amounts of the adherent yeast form of *C. glabrata* detected by the washing out method are shown in Fig. 4a. In the control group and 0.1 mg/mL of mannan, a large amount of *C. glabrata* biofilms was observed, and the CFU level was almost the same level as that of *C. albicans* (Fig. 4a). There was a significant difference between the 10 mg/mL of mannan group and the control groups or the 0.1 mg/mL of mannan group ($p < 0.05$), but there were no significant differences among the mannan groups.

Inhibition of the adherence of *C. glabrata* by mannan, as determined by the REDOX indicator assay

In the control group, the saliva group and the mannose group, a large amount of *C. glabrata* biofilms was observed, and the fluorescence level was almost the same

Fig. 3 The duration effect of Mannan by measuring the amount of hyphal form of *C. albicans* adhesion to acrylic surfaces coated with mannan.

Statistical analyzes were performed among multigroups in a day or multigroups in concentration by Kruskal-Wallis, and between each groups by Mann-Whitney U-test (Significant differences were judged as $p < 0.05$). In the first and second day, all test groups in amount of fluorescence were significant different except for between 0.1 mg/mL and 1 mg/mL of mannan in the second day. In the third day, control, 0.1 mg/mL and 1 mg/mL of mannan were significantly different between 10 mg/mL of mannan ($p < 0.05$).

level as that of *C. albicans* (Fig. 4b). The fluorescence levels of *C. glabrata* that adhered to the pieces treated with 0.1 mg/mL of mannan was 62% the level of the control group, and was 40% for the pieces treated with 1 mg/mL, and 5% for samples treated with 10 mg/mL. There was a significant difference between the 10 mg/mL of mannan group and the control, the saliva and mannose groups ($p < 0.05$), but there were no significant differences among the mannan groups.

DISCUSSION

The outermost layer of the cell wall of *Candida* consists of a complex of hydrophilic polysaccharides, such as galactomannan and mannan, which are involved in the adhesion of the *Candida* to host cells, as well as the antigenicity^{11,12}. Iwamoto *et al.* reported that mannan from *S. cerevisiae* had an inhibitory effect on the adhesion of *C. albicans* to plastic culture dish surfaces^{13,14}. They examined only the adhesion inhibitory effect for the hyphal form of *C. albicans*, not the yeast form. The biofilms of *C. albicans* consist of both the yeast and hyphal forms, therefore, inhibiting the adhesion of both forms is important^{3,20}.

Recently, *C. glabrata* was not only reported to have a high detection rate in the oral cavity of elderly subjects, but was also identified as a pathogen causing denture stomatitis. *C. glabrata* frequently acquires

Fig. 4 The amount of adhered *C. glabrata* to mannan treated resin surfaces.

a: The detection by washing out method

There was significantly difference between two groups. (Mann-Whitney U-test, $p < 0.05$).

b: The detection by Redox indicator assay

Statistical analyzes were performed among multigroups by Kruskal-Wallis, and between two groups of the control group and each test group was analyzed by Mann-Whitney U-test, significant differences were judged as $p < 0.05$.

resistance to antifungal drugs, making it difficult to eradicate²¹. In this study, we examined the inhibitory effect of a mannan coating on the denture base acrylic surface against the adhesion of both of *C. albicans* and *C. glabrata*. In addition, since it is important to know how long the inhibitory effect of the mannan coating will be maintained for clinical applications, we carried out a durability test of the mannan against *C. albicans* adhesion.

The adhesion of the yeast form of *C. albicans* was inhibited in a mannan concentration-dependent manner, as indicated by experiments using a modified JIS Z 2801 method (Fig. 1). Since the yeast form of *C. albicans* often switches to the hyphal form in the oral cavity²⁰, we also

wanted to examine the effects on the hyphal form of *C. albicans*. However, the hyphal form of *C. albicans* could not be recovered by the washing out method even in the control group, because *C. albicans* was too strongly attached on the surface of the denture base acrylic pieces to be released into the washing medium (data not shown). Therefore, the detection system was changed to a method monitoring the viable microbial count by examining the metabolism using the REDOX indicator. As the REDOX indicator in the medium measures the metabolism based on the amount of fluorescence produced, it reflects the number of surviving microorganisms²²⁾.

From the results shown in Figs. 2a and 4b, it can be concluded that mannan inhibited the adhesion of *Candida* in a concentration-dependent manner, but mannose was not able to inhibit *Candida* adhesion even at a high concentration. Iwamoto *et al.* reported that the adhesion inhibitory effect was observed after coating the surface with 1 mg/mL of mannan for two hours, but that there was not an inhibitory effect for 0.1 mg/mL of mannan¹⁴⁾. In this study, the application of 0.1 mg/mL of mannan coating overnight showed an inhibitory effects on the adhesion of the hyphal form of *C. albicans* (Fig. 2a). The difference between these results was likely due to the coating time.

In the case of *C. glabrata*, the inhibitory effect was also observed to occur in a concentration-dependent manner (Figs. 4a and 4b), and the 10 mg/mL of mannan led to significantly higher anti-adhesive effects. This indicates that mannan effectively prevents the adhesion of two major *Candida* species to the denture surface, indicating the possibility of applying such a coated for clinical dentistry.

Recently, mannan binding molecules on the surface dendritic cells, Dectin-2 and DC-sign, were found²³⁻²⁶⁾. In dendritic cells and macrophages, the expression of inflammatory cytokines such as TNF- α is induced by the binding of the mannoooligosaccharide in mannan^{10,15)}. However, dendritic cells are generally not exposed to the outermost layer of the oral mucosa. Therefore, no immune response should be induced in the oral cavity due to its separation by the epithelium, and thus, inflammation should not be induced by coating denture surfaces with mannan. Additionally, there were some contradictory reports about the role of mannan in inflammation. For example, one report indicated that mannan promotes wound healing by increasing the proliferation of fibroblasts and vascular endothelial cells, and by enhancing the production of collagen¹⁶⁾, and foods and medical devices including mannan have been developed based on these properties.

Although there is much demand for acrylic resin base dentures in aging societies, they are difficult to keep clean. Mannan has been applied widely in the medical field, particularly for the prevention of influenza and bacterial infections, inflammation and allergic diseases²⁷⁻²⁹⁾. Based on the previous results and those from the present study, we advocate the use of mannan in the clinical practice of dentistry, as employing a mannan coating on dental devices might improve the

oral hygiene. However, in this study, the inhibitory effect of the mannan coating on denture resin pieces against *Candida* adhesion lasted for three days. It is therefore necessary to investigate methods to stabilize mannan, such as the cross-linking between mannan molecules and the denture base acrylic pieces, in the future.

In conclusion, our results suggest that applying a mannan coating on the denture base acrylic resin can prevent the adhesion of *C. albicans* and *C. glabrata*.

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