Saccharomyces boulardii Protease Inhibits the Effects of Clostridium difficile Toxins A and B in Human Colonic Mucosa

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Saccharomyces boulardii is a nonpathogenic yeast used in the treatment of Clostridium difficile diarrhea and colitis. We have reported that S. boulardii inhibits C. difficile toxin A enteritis in rats by releasing a 54-kDa protease which digests the toxin A molecule and its brush border membrane (BBM) receptor (I. Castagliuolo, J. T. Lamont, S. T. Nikulasson, and C. Pothoulakis, Infect. Immun. 64:5225–5232, 1996). The aim of this study was to further evaluate the role of S. boulardii protease in preventing C. difficile toxin A enteritis in rat ileum and determine whether it protects human colonic mucosa from C. difficile toxins. A polyclonal rabbit antiserum raised against purified S. boulardii serine protease inhibited by 73% the proteolytic activity present in S. boulardii conditioned medium in vitro. The anti-protease immunoglobulin G (IgG) prevented the action of S. boulardii on toxin A-induced intestinal secretion and mucosal permeability to [3H]mannitol in rat ileal loops, while control rabbit IgG had no effect. The anti-protease IgG also prevented the effects of S. boulardii protease on digestion of toxins A and B and on binding of [3H]toxin A and [3H]toxin B to purified human colonic BBM. Purified S. boulardii protease reversed toxin A- and toxin B-induced inhibition of protein synthesis in human colonic (HT-29) cells. Furthermore, toxin A- and B-induced drops in transepithelial resistance in human colonic mucosa mounted in Ussing chambers were reversed by 60 and 68%, respectively, by preexposing the toxins to S. boulardii protease. We conclude that the protective effects of S. boulardii on C. difficile-induced inflammatory diarrhea in humans are due, at least in part, to proteolytic digestion of toxin A and B molecules by a secreted protease.

Clostridium difficile is the causative agent of antibiotic-associated colitis in humans and animals (1, 2). Following antibiotic intake by animals and humans, C. difficile colonizes the intestine and releases two potent protein exotoxins, toxin A and toxin B, which mediate diarrhea and colitis caused by this microbe (16, 20, 24). Although both toxins A and B are potent cytotoxins (20, 27, 15, 32) and induce release of inflammatory mediators from immune cells in vitro (19), only toxin A possesses enterotoxic effects in rodent intestine (38). Injection of toxin A into rat intestinal loops causes fluid secretion, increased mucosal permeability, mucosal damage (7, 17, 38), and release of inflammatory mediators from lamina propria immune cells (8, 9). However, a recent in vitro study showed that toxin B and to a lesser extent toxin A are able to cause tissue damage and electrophysiologic changes in normal human colon in vitro (32), suggesting that both C. difficile toxins are involved in the pathophysiology of human colitis.

Saccharomyces boulardii, a nonpathogenic yeast, is effective in the prevention and treatment of many forms of diarrhea in humans, especially antibiotic-associated diarrhea and colitis (6, 25, 36). Recent studies showed that S. boulardii administration significantly reduced the frequency of diarrhea in patients administered antibiotic therapy and that in combination with vancomycin or metronidazole it reduced the number of relapses of C. difficile infection (26). The mechanism by which S. boulardii mediates its protective intestinal effects has been investigated (10, 11, 12, 13, 22, 37). We previously reported that oral administration of S. boulardii to rats diminished ileal fluid secretion and mucosal damage in response to intraluminal administration of purified toxin A (29). Subsequently, we reported that these protective effects of S. boulardii in rat ileum appeared to be mediated by a 54-kDa serine protease which cleaves toxin A and its intestinal receptor (7).

The present study was undertaken to further elucidate the role of the 54-kDa S. boulardii protease in toxin A-mediated enteritis in rat ileum with a polyclonal antibody directed against the purified S. boulardii protease. We also determined whether this protease has a role in protecting the human colon from the effects of C. difficile toxins A and B. We demonstrate here that toxin A- and B-induced electrophysiologic and cytotoxic effects in human colon are also markedly attenuated by preincubating the C. difficile toxins A and B with purified S. boulardii protease prior to addition to human colonic mucosa.

MATERIALS AND METHODS

Male Wistar rats weighing 200 to 250 g were obtained from Charles River Breeding Laboratories (Wilmington, Mass.). Before the experiments rats were fasted overnight but had free access to water. New Zealand White rabbits used to generate antisera against S. boulardii protease were obtained from Harer-Marland Laboratories (Hewit, N.J.). Pentobarbital sodium (Nembutal; 50 μg/ml) was obtained from Abbott (North Chicago, Ill.), Sabouraud dextrose broth for culturing S. boulardii was obtained from Difco (Detroit, Mich.). A bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.) was used for measuring protein concentrations. The Bolton-Hunter reagent for toxin labeling (N-succinimidyl [2,3-3H]propionate, 80 Ci/mmol) was purchased from Amersham International (Amersham, England). Fetal calf serum and other tissue culture supplies were obtained from Sigma Diagnostics (St. Louis, Mo.).

Toxin A and B purification and radiolabeling. Toxins A and B were purified from culture supernatants of C. difficile VPI strain 10463 (American Type Culture Collection, Rockville, Md.) as previously described (7–9, 27). Toxin A and toxin B were radiolabeled with tritium with the Bolton-Hunter reagent as previously described by us (30). Both irradiated toxins retained their cytotoxic activity.
Effect of anti-S. boulardii protease IgG on the proteolytic activity of S. boulardii conditioned medium. S. boulardii conditioned medium (50 μg/ml) was incubated (22°C) with anti-S. boulardii protease or control rabbit IgG. After 1 h of incubation, the proteolytic activity present in the conditioned medium was determined by the method of Roth et al. (34) as previously described by us (7). Briefly, the mixtures were incubated (37°C) with the nonspecific protease substrate [methyl-14C]methemoglobin (1 μCi/ml) or purified protease (0.1 μg/ml). After the plates were washed four times with PBS-T, goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5,000) was added and the mixture was incubated for 1 h at 37°C. Wells were then washed six times, and 50 μl of peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) was added to each well. The reaction was stopped after 5 to 10 min by addition of PHO3 (1 M), and absorbance at 450 nm was then recorded.

Effect of anti-S. boulardii protease IgG on the proteolytic activity of S. boulardii conditioned medium. S. boulardii conditioned medium (3.0 mg in 200 μl) was incubated (22°C for 1 h) with anti-S. boulardii protease IgG, control rabbit IgG (1:1,000 for both), or saline alone. At the end of the incubation period 5 μg of toxin A in 50 mM Tris buffer or buffer alone was added and incubated for an additional hour at 37°C. A midline abdominal incision was performed on anesthetized rats as previously described, and two 5-cm-long closed ileal loops were formed (7, 29). Renal excretion of [3H]mannitol was prevented by closing the renal pedicles with silk, and [3H]mannitol (10 μCi) was injected intraperitoneally. Each ileal loop was then injected (40 μl) with one of the toxins A or S. boulardii mixtures described above, and was then incubated for 30 min at 37°C with or without the S. boulardii conditioned medium (0.1 mg/ml) or purified protease (0.1 μg/ml) in 1 ml of 50 mM Tris buffer (pH 7.4). The reaction was halted by adding 0.2 ml of 50% TCA, and samples were placed on ice for 30 min. Samples were then centrifuged (700 × g for 30 min at 4°C), and 0.25-ml aliquots of the supernatant were measured for radioactivity content by scintillation counting. In similar experiments, the effect of S. boulardii protease IgG on digestion of toxins was assessed by preincubating S. boulardii conditioned medium with the rabbit polyclonal anti-S. boulardii protease IgG as described above.

Effect of anti-S. boulardii protease IgG on S. boulardii-mediated inhibition of the ability of H-toxin A and H-toxin B to bind to human colonic BBM. Animals were injected subcutaneously (250 μl per g) in 0.2 M acetate buffer (pH 8.0) with 1 ml of 0.2 M acetate buffer containing either S. boulardii protease (1 to 10 μg/ml) or purified protease (0.1 μg/ml). After the plates were washed four times with PBS-T, goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5,000) was added and the mixture was incubated for 1 h at 37°C. Wells were then washed six times, and 50 μl of peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) was added to each well. The reaction was stopped after 5 to 10 min by addition of PHO3 (1 M), and absorbance at 450 nm was then recorded.

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Effect of anti-S. boulardii protease IgG on S. boulardii-conditioned medium. S. boulardii conditioned medium was reconstituted in Sabouraud dextrose broth (1 g in 10 ml of medium) and cultured at 37°C as previously described (7, 29). S. boulardii conditioned medium was obtained after 48 h by centrifuging the yeast culture (1,000 × g for 10 min at 4°C) and filtering the supernatant through a 0.2-μm-pore-size filter. S. boulardii protease was purified as previously described by us (7). Briefly, S. boulardii conditioned medium was concentrated 5-fold on a Amicon PM-10 filter (Gelman Scientific), size fractionated on a G-50 gel filtration column (Sigma), and finally purified on a Octyl-Sepharose CL-4B column (Pharmacia Biotech, Uppsala, Sweden).

Preparation of polyclonal antibodies against purified S. boulardii serine protease, S. boulardii protease, purified as described above, was used to immunize adult male rabbits. Ten milligrams of the antigen in 10 mM Tris buffer was mixed 1:1 (vol/vol) with adjuvant (Ribi Immunocore Inc., Hamilton, Mont.) in distilled water according to the manufacturer’s recommendations. Animals were injected subcutaneously (250 μl per g) with the protease-adjuvant mixture every 2 weeks for a period of 4 weeks and then every month for 2 months. One rabbit was immunized with vehicle plus adjuvant alone (control). Sera were collected 7 days after the last two injections, and the immunoglobulin G (IgG) fractions were purified on a protein A-Sepharose column (Pharmacia Biotech). Titters of anti-S. boulardii protease antibodies were determined in vitro by enzyme-linked immunosorbent assay. Fifty microliters of either S. boulardii conditioned medium (1 to 10 μg/ml) or purified S. boulardii protease (10 μg/ml) was first incubated (1 h at 37°C) with anti-S. boulardii protease IgG or control rabbit IgG. After the plates were washed four times with PBS-T, goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5,000) was added and the mixture was incubated for 1 h at 37°C. Wells were then washed six times, and 50 μl of peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) was added to each well. The reaction was stopped after 5 to 10 min by addition of PHO3 (1 M), and absorbance at 450 nm was then recorded.

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RESULTS

Anti-S. boulardii protease IgG inhibits proteolytic activity in S. boulardii conditioned medium. We previously reported that conditioned media of S. boulardii cultures possess proteolytic activity against [14C]methemoglobin substrate (7). We first determined the effect of the anti-S. boulardii protease antiserum on the proteolytic activity present in the S. boulardii conditioned medium. Incubation of S. boulardii conditioned medium with [14C]methemoglobin caused a significant increase in radioactivity released in the TCA supernatant after a 20-min incubation (Fig. 1). Preincubation of S. boulardii conditioned medium with a 10−3 dilution of anti-S. boulardii protease IgG

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inhibited proteolytic activity by 73%, whereas similar dilution of control rabbit IgG had no significant inhibitory effect (Fig. 1). Addition of a $2 \times 10^{-4}$ dilution of the anti- \textit{S. boulardii} protease IgG inhibited proteolytic activity of \textit{S. boulardii} conditioned medium by 49%, whereas a $10^{-4}$ dilution had no significant inhibitory effect (Fig. 1), indicating that the effect of anti-\textit{S. boulardii} protease is dose dependent.

\textbf{Anti-\textit{S. boulardii} protease IgG reverses the protective effects of \textit{S. boulardii} in toxin A-mediated enteritis.} We next determined the action of anti-\textit{S. boulardii} protease IgG on the protective effects of \textit{S. boulardii} conditioned medium in toxin A-induced enteritis in rat ileum in vivo. As expected (7, 8, 29, 38), injection of purified toxin A into rat ileal loops increased fluid secretion and mucosal permeability to mannitol after 4 h of exposure compared to levels produced in buffer-injected loops (Fig. 2). As previously reported (7, 29), preincubation of toxin A with \textit{S. boulardii} conditioned medium for 30 min in vitro significantly inhibited toxin A-induced ileal secretion and mucosal permeability (Fig. 2). However, the protective effect of \textit{S. boulardii} conditioned medium on toxin A-induced ileal responses was significantly reversed when \textit{S. boulardii} conditioned medium was preincubated with the rabbit polyclonal IgG directed against the 54-kDa \textit{S. boulardii} protease (Fig. 2). Preincubation of \textit{S. boulardii} conditioned medium with control rabbit IgG had no effect on the inhibitory effects of \textit{S. boulardii} in toxin A-mediated fluid secretion and mannitol permeability (Fig. 2).

\textit{S. boulardii}-mediated proteolysis of \textit{C. difficile} toxin A and toxin B is inhibited by anti-\textit{S. boulardii} protease IgG. We previously reported that the 54-kDa serine protease from \textit{S. boulardii} digests toxin A in vitro (7). Riegler et al. (32) recently reported that both toxin A and toxin B diminished mucosal resistance and caused epithelial cell damage in human colonic mucosa in vitro. Accordingly, we sought to determine whether \textit{S. boulardii} protease could also digest \textit{C. difficile} toxin B. As shown in Table 1, both \textit{S. boulardii} conditioned medium and its purified protease digested $^{3}$H-toxin A and $^{3}$H-toxin B. In order to confirm that the effects of \textit{S. boulardii} protease on toxins A and B were due to proteolysis of the toxin molecules, the nonspecific protease inhibitor a2-macroglobulin was incubated with purified protease and their proteolytic activity on tritiated toxins A and B was determined. Our results show that preincubation with a2-macroglobulin completely prevented digestion of both toxins by \textit{S. boulardii} protease (Table 1). In addition, preincubation of \textit{S. boulardii} conditioned medium with anti-\textit{S. boulardii} protease IgG inhibited digestion of both $^{3}$H-toxin A and $^{3}$H-toxin B by 40 and 63%, respectively ($P < 0.01$; Table 1).

\textbf{Anti-\textit{S. boulardii} protease IgG prevents the action of \textit{S. boulardii} on the ability of $^{3}$H-toxin A and $^{3}$H-toxin B to bind to human colonic BBM.} We previously reported that exposure of $^{3}$H-toxin A to \textit{S. boulardii} protease results in diminished $^{3}$H-toxin A binding to its rat BBM receptor(s) (7). Since previous results indicated the presence of functional toxin A and toxin B receptors on human colon (32), we tested the effect of \textit{S. boulardii} protease on toxin A and B binding to human colonic BBM receptors. As expected $^{3}$H-toxin A and $^{3}$H-toxin B bound to human colonic BBM, and approximately 60% of the binding was inhibited by a 1,000-fold excess of unlabeled toxins (data not shown). Incubation of $^{3}$H-toxin A or B with purified \textit{S. boulardii} protease significantly reduced $^{3}$H-toxin A and B bind-
ing to human colonic BBM by 60 and 58%, respectively (Table 2). A less substantial inhibition was observed when BBM were first incubated with purified protease before addition of radiolabeled toxins A and B (data not shown), suggesting that the S. boulardii protease may also digest the human colonic toxin A and B receptors.

* S. boulardii protease prevents toxin A- and B-induced inhibition of protein synthesis in HT-29 cells. Since previous studies from our laboratory indicated that toxin B inhibited protein synthesis in human fibroblasts and hamster cecal explants (31), we next determined the effect of purified S. boulardii protease on toxin A- and B-induced inhibition of protein synthesis in a human colonic adenocarcinoma (HT-29) cells. Our results showed that incubation of HT-29 cells with 1 μg of toxin A or B per ml resulted in a significant inhibition of protein synthesis after 16 h of incubation (Fig. 3). However, preexposure of toxin A and toxin B to the S. boulardii protease significantly reduced inhibition of protein synthesis caused by the toxins (Fig. 3). In contrast, protein synthesis in HT-29 cells incubated with the S. boulardii protease alone was no different from that of buffer-exposed cells (Fig. 3).

**S. boulardii protease inhibits toxin A- and toxin B-mediated reduction in tissue resistance of human colonic mucosa mounted in Ussing chambers.** We recently reported that toxin A and B reduced tissue resistance in human colon in vitro (32). We next tested the ability of S. boulardii protease to inhibit this toxin-mediated colonic effect. Normal colonic mucosa incubated with either buffer alone or buffer containing S. boulardii protease for 3.5 h showed stable electrophysiological values (Fig. 4). Exposure to either toxin A or toxin B caused a significant drop in colonic transepithelial resistance during the 3.5-h incubation period. Preincubation of toxin A and B with S. boulardii protease prevented the actions of the toxins on transepithelial resistance (Fig. 4).

**DISCUSSION**

Our previous studies suggested that a 54-kDa serine protease purified from conditioned medium of S. boulardii cultures mediates the inhibitory action of the yeast on C. difficile toxin A enteritis in rats by digesting the toxin A molecule and by inhibiting toxin A binding to its surface BBM enterocytte receptor (7, 29). Since both C. difficile toxins A and B exert direct effects on human colonic cell lines and native human colonic mucosa (3, 21), we studied here the action of the S. boulardii protease on the toxin B molecule and on toxin A- and

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<th>Tested mixture</th>
<th>3H-toxin A + buffer (control)</th>
<th>3H-toxin A + S. boulardii CM</th>
<th>3H-toxin A + anti-protease IgG</th>
<th>3H-toxin B + buffer (control)</th>
<th>3H-toxin B + S. boulardii CM</th>
<th>3H-toxin B + anti-protease IgG</th>
<th>3H-toxin B + control IgG</th>
<th>3H-toxin B + S. boulardii CM + control IgG</th>
<th>3H-toxin B + S. boulardii CM + anti-protease IgG</th>
<th>3H-toxin B + S. boulardii CM + control IgG</th>
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<tr>
<td>Binding</td>
<td>140 ± 40</td>
<td>8,730 ± 200**</td>
<td>3,370 ± 350**</td>
<td>7,750 ± 380**</td>
<td>3,500 ± 250**</td>
<td>2,200 ± 70</td>
<td>8,850 ± 310**</td>
<td>5,880 ± 420**</td>
<td>9,150 ± 390**</td>
<td>4,200 ± 240**</td>
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*Results are the means ± standard errors of the means of three separate experiments performed for each experimental approach, with duplicate determinations made for each. 3H]Toxin A or 3H]Toxin B (0.1 μg containing 40,000 dpm) was first incubated (30 min at 37°C) with S. boulardii conditioned medium (CM; 100 μg). The mixture was then added to purified human colonic BBM (50 μg/0.2 ml), and 3H]Toxin A- or B-specific binding to BBM was measured as described in Materials and Methods. **, P < 0.01 versus values for the corresponding controls. ***, P < 0.01 versus values for 3H]toxin binding in the presence of S. boulardii conditioned medium.
S. boulardii protease prevents toxin A- and B-mediated inhibition of protein synthesis in colonic epithelial (HT-29) cells. Purified toxin A or B (1 μg/ml) was incubated (1 h at 37°C) with either purified S. boulardii conditioned medium (100 μg/ml) or buffer alone before addition to cultured HT-29 cells (10⁶ cells per well). After 4 h [³H]leucine (3 μCi/ml) was added to the culture media and the mixtures were incubated for an additional 16 h. At the end of the incubation period, culture media and cells were collected separately and proteins were precipitated by addition of TCA. Radioactivity contents in the precipitated proteins were measured as an indicator of protein synthesis and expressed as disintegrations per minute per well. Results are expressed as means ± standard errors of the means of results for each group; four to six wells were tested for each experimental condition, and duplicate determinations were made for each. **P < 0.01 versus values for the control; *P < 0.01 versus values for toxin A or B alone. Tx: toxin; Sb: S. boulardii.

Our results indicate that a polyclonal antibody directed against the 54-kDa S. boulardii protease reversed the proteolytic activity of S. boulardii conditioned medium against toxins A and B (Fig. 1) and the inhibition of radioactively labeled toxin A and B binding to human colonic BBM mediated by S. boulardii (Table 2). Most importantly, the anti-S. boulardii protease antibody also almost completely reversed the inhibitory effect of S. boulardii conditioned medium on fluid secretion and mucosa permeability observed after administration of toxin A in rat ileum in vivo (Fig. 2). These results confirm and extend our earlier observations that the S. boulardii protease mediates a large part of the yeast’s effects against C. difficile ([7, 29]).

We recently demonstrated that only luminal, and not basolateral, administration of C. difficile toxins induces dose-dependent damage of surface, but not crypt, colonocytes (32, 33) and that functional toxin A receptors are localized in the apical membrane (32). Furthermore, toxin-mediated damage is accompanied by a reduction in transepithelial resistance (15, 32). In keeping with these observations, results in the present study demonstrate that toxins A and B induce a reduction of epithelial barrier integrity (Fig. 4) probably via binding to specific receptors on surface colonocytes. Indeed, using tritiated toxins A and B we demonstrated here the presence of specific receptors for both toxins on human colonic BBM (Table 2). In addition, incubation of toxins A and B with S. boulardii conditioned medium and purified S. boulardii protease reversed a toxin-mediated drop in tissue resistance (Fig. 4) and reduced toxin receptor binding to human BBM (Table 2). Since both S. boulardii and its 54-kDa protease digest toxin A and toxin B, we conclude that the protective effects of S. boulardii on toxin A- and B-induced colonic responses are mediated by proteolytic cleavage of the toxins.

Although results in this and our previous studies (7, 29) point to a major role for the S. boulardii serine protease in the protective effects of the yeast in C. difficile colitis, several other proposed mechanisms may also account for the protective effects of S. boulardii in this infection. These include factors produced by S. boulardii in vivo which may inhibit C. difficile growth and cause reduced toxin production (10, 14). In addition, the ability of S. boulardii to stimulate host mucosal disaccharidase activity (4) and enhance the intestinal mucosal immune response (5) may also be involved in the mechanism by which S. boulardii reduces the recurrence of C. difficile colitis.

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