

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 antiserum against *S. boulardii* protease were obtained from Hare-Marland Laboratories (Hewitt, 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 biconchonic 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 tritiated toxins retained their cytotoxic activity

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against rabbit lung R9ab fibroblasts (30). Purity of unlabeled and labeled toxins was assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (18). Purified toxin A and toxin B preparations contained single protein bands at ~300 and 270 kDa, respectively.

Purification of *S. boulardii* protease. *S. boulardii* was provided from Biocodex Laboratories (Montrouge, France) as a lyophilized powder. *S. boulardii* 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 fivefold on an Amicon PM-50 filter (Gelmans 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 micrograms of the antigen in 10 mM Tris buffer was mixed 1:1 (vol/vol) with adjuvant (Ribi ImmunoChem Research, Inc., Hamilton, Mont.) in distilled water according to the manufacturer's recommendations. Animals were injected subcutaneously (250 μl per thigh) 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). Titers of anti-*S. boulardii* protease antibodies were determined *in vitro* by enzyme-linked immunosorbent assay. Fifty microliters of either *S. boulardii* conditioned medium (50 to 1 μg/well), purified *S. boulardii* protease (1 to 10 μg/well), or trypsin from bovine pancreas (10 μg/well; Sigma), which served as a protease control, was dissolved in carbonate buffer (pH 8.0), added onto 96-well plates, and incubated for 16 h at 4°C. Wells were then washed four times with 1× phosphate-buffered saline (PBS) containing 0.25% Tween 20 (PBS-T), blocked (2 h at 37°C) in PBS-T containing 2% bovine serum albumin, and then 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.

Effect of anti-*S. boulardii* protease IgG on the proteolytic activity of *S. boulardii* conditioned medium. *S. boulardii* conditioned medium (50 μg) 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 μg, 50,000 dpm) in 1 ml of 0.2 M acetate buffer (pH 3.8) in a shaking water bath. After 20 min, the reaction was terminated by adding 0.2 ml of 50% trichloroacetic acid (TCA) and cooling the samples on ice. The reaction mixtures were then centrifuged (700 × g for 30 min at 4°C), and 0.25 ml of the supernatant containing small methemoglobin peptides released by proteolytic digestion was assayed for radioactivity by scintillation counting.

Effect of anti-*S. boulardii* protease IgG on *S. boulardii*-mediated protection of *C. difficile* toxin A enteritis. To further ascertain the role of *S. boulardii* serine protease in toxin A enteritis, we studied the effect of anti-*S. boulardii* protease IgG on the protective effects of *S. boulardii* in toxin A-induced enteritis in rats. *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 intravenously. Each ileal loop was then injected (400 μl) with one of the toxin A-*S. boulardii* mixtures described above, the abdominal incision was sutured, and animals were kept under light anesthesia while the body temperature was maintained at 37°C by a heating pad. After 4 h animals were sacrificed by an intraperitoneal bolus of pentobarbital and the loops were removed. Intestinal permeability was determined as blood-to-lumen excretion of [³H]mannitol, and results were expressed as disintegration of [³H]mannitol per minute per centimeter of loop as previously described (7, 8, 29, 38). Intestinal fluid secretion was measured as the ratio of loop weight (milligrams) to length (centimeter) as previously described (7, 8, 38). The study was approved by the Beth Israel and Deaconess Medical Center Institutional Animal Care and Use Committee.

Effect of anti-*S. boulardii* protease IgG on *S. boulardii*-mediated proteolytic activity against purified *C. difficile* toxins A and B. We previously reported that *S. boulardii* protease digests toxin A *in vitro* (7). However, since both toxin A and toxin B damage human colonic mucosae *in vitro* (32), we examined whether *S. boulardii* protease also digests toxin B. The effect of *S. boulardii* protease on *C. difficile* toxins A and B was determined by a modification (7) of the method of Roth et al. (34). Tritiated toxin A or B (0.1 μg containing 40,000 dpm) was

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 ³H-toxin A and ³H-toxin B to bind to human colonic BBM. Human colonic brush border membranes (BBM) were prepared from normal colonic segments obtained from surgical resections as previously reported by us (28). Binding of ³H-toxin A and ³H-toxin B to purified human colonic BBM was measured as described previously (28). *S. boulardii* conditioned medium (100 μg) or medium alone (control) was incubated with anti-*S. boulardii* protease IgG, control rabbit IgG, or vehicle alone (1 h at 22°C). ³H-toxin A or ³H-toxin B (0.1 μg in 10 μl containing 40,000 dpm) was added and further incubated for 60 min at 37°C. At the end of the incubation the ³H-toxin A- and ³H-toxin B-anti-*S. boulardii* protease IgG mixtures were added to human colonic BBM (50 μg/tube) and incubated (1 h at 22°C) in a final volume of 200 μl of 50 mM Tris buffer (pH 7.4). After incubation, BBM were washed twice with 1 ml of ice-cold 50 mM Tris buffer (pH 7.4) to remove unbound toxin. Pellets were dissolved in 0.2 ml 10% sodium dodecyl sulfate, and BBM-associated radioactivity was determined by scintillation counting. Specific ³H-toxin A and ³H-toxin B binding was calculated as described previously (7, 28, 30). Background radioactivity in tubes containing ³H-toxin A or ³H-toxin B but no membranes was subtracted.

Effect of *S. boulardii* protease on toxin A- and B-induced inhibition of protein synthesis in HT-29 cell monolayers. The effect of purified *S. boulardii* protease on *C. difficile* toxin A- and B-mediated inhibition of protein synthesis in human colonic adenocarcinoma (HT-29) cells was determined by a modification of the method described by McClane and McDonell (23) and previously described by us (31). HT-29 cells (American Type Culture Collection) were grown in 250-ml Falcon tissue culture flasks (Becton Dickinson, Lincoln Park, N.J.) and seeded (10⁵ cells/ml) onto 12-well tissue culture plates for 24 h in Dulbecco modified Eagle medium containing 10% heat inactivated fetal calf serum. Purified toxin A or B (1 μg/ml) was first incubated (1 h at 37°C) with either purified *S. boulardii* conditioned medium (100 μg/ml) or buffer alone. These mixtures were then added to the HT-29 monolayers and incubated for 4 h at 37°C. [³H]leucine (3 μCi/ml) was then 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 transferred to glass tubes, and proteins were precipitated by addition of 10% TCA (18 h at 4°C). The mixtures were centrifuged (2,000 × g at 4°C for 30 min), and pellets were washed twice with 10% TCA and finally resuspended in 0.5 ml of 0.3 M NaOH. Radioactivity was counted in 0.2-ml aliquots to determine incorporation of [³H]leucine as an indicator of cellular protein synthesis (31).

Effect of *S. boulardii* protease on toxin A- and B-induced resistance changes of human colonic mucosae in Ussing chambers. Human colonic tissues were obtained from colonic resections. A total of 20 tumor-free specimens of human left-sided colon were used in these experiments. Human and rabbit colonic specimens were opened longitudinally, the muscle layers were dissected under the microscope, and up to six colonic mucosal preparations from each specimen (1.0-cm² surface area) were mounted in Ussing chambers (DCTSYS; Precision Instrument Design, San Diego, Calif.) (32). Luminal and serosal sides were bathed at 37°C with 7 ml of nutrient buffer as previously described (32) and left to equilibrate for 1 h. At the end of the equilibration period the buffer on the apical side was replaced with either fresh nutrient buffer alone (control) or buffer containing toxin A or B (10 μg/ml). In some experiments, purified toxin A (32 nM), toxin B (3 nM), or buffer alone was incubated (1 h, 37°C) with *S. boulardii* protease (1 μg/ml) prior to placement in the Ussing chambers. Changes in electrical resistance (ohms per square centimeter) were recorded every 30 min over a 4-h period as previously described (32). The protocol for use of human tissues was approved by the Ethics Committee of Beth Israel Deaconess Medical Center.

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

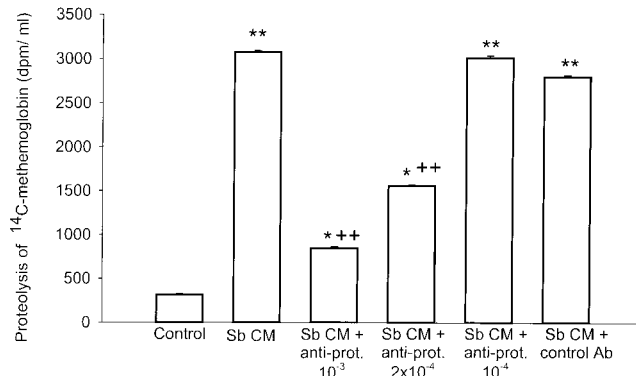


FIG. 1. Effect of anti-*S. bouhardii* protease IgG on *S. bouhardii* protease activity against [¹⁴C]methemoglobin. *S. bouhardii* conditioned medium (CM) (50 µg) or medium alone was incubated (1 h at 22°C) with different dilutions of rabbit anti-*S. bouhardii* protease polyclonal IgG or with control rabbit IgG. To determine the proteolytic activity in the *S. bouhardii* CM, the mixtures were incubated at 37°C with [methyl-¹⁴C]methemoglobin. After 20 min the reactions were terminated by adding TCA, and samples were centrifuged. To determine the proteolytic activity, the radioactivity released in the supernatant was measured by scintillation counting. Results are the means ± standard errors of the means of results from three to six separate experiments, each with duplicate determinations. **, $P < 0.01$, and *, $P < 0.05$ versus values for respective controls; +, $P < 0.01$ versus values for *S. bouhardii* CM alone. Ab, antibody; prot., protease; Sb, *S. bouhardii*.

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

Anti-*S. bouhardii* protease IgG reverses the protective effects of *S. bouhardii* in toxin A-mediated enteritis. We next determined the action of anti-*S. bouhardii* protease IgG on the protective effects of *S. bouhardii* 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 *S. bouhardii* conditioned medium for 30 min in vitro significantly inhibited toxin A-induced ileal secretion and mucosal permeability (Fig. 2). However, the protective effect of *S. bouhardii* conditioned medium on toxin A-induced ileal responses was significantly reversed when *S. bouhardii* conditioned medium was preincubated with the rabbit polyclonal IgG directed against the 54-kDa *S. bouhardii* protease (Fig. 2). Preincubation of *S. bouhardii* conditioned medium with control rabbit IgG had no effect on the inhibitory effects of *S. bouhardii* in toxin A-mediated fluid secretion and mannitol permeability (Fig. 2).

***S. bouhardii*-mediated proteolysis of *C. difficile* toxin A and toxin B is inhibited by anti-*S. bouhardii* protease IgG.** We previously reported that the 54-kDa serine protease from *S. bouhardii* 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 *S. bouhardii* protease could also digest *C. difficile* toxin B. As shown in Table 1, both *S. bouhardii* conditioned medium and its purified protease digested ³H-toxin A and ³H-toxin B. In order to confirm that the effects of *S. bouhardii* protease on toxins A

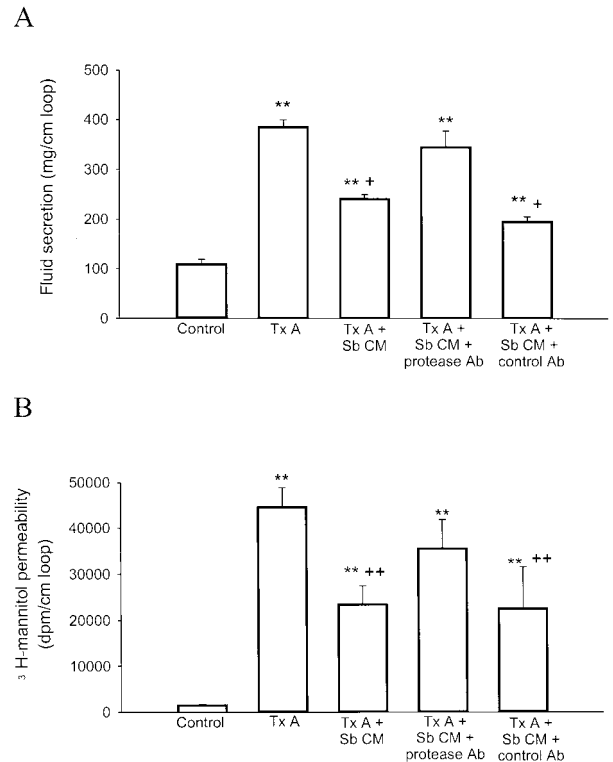


FIG. 2. Anti-*S. bouhardii* protease IgG reverses the protective effects of *S. bouhardii* in toxin A-mediated enteritis. Rat ileal loops were injected with 5 µg of toxin A or toxin A preincubated (1 h at 37°C) with *S. bouhardii* conditioned medium (3.0 mg in 200 µl). In other experiments *S. bouhardii* conditioned medium was incubated (22°C for 1 h) with anti-*S. bouhardii* protease IgG or control rabbit IgG (dilution, 1:1,000 for both) before ileal toxin A administration as described above. After 4 h animals were sacrificed and secretion of fluid was measured as the ratio of loop weight (micrograms) to length (centimeters) (A). [³H]mannitol permeability was expressed as disintegration of [³H]mannitol per minute per centimeter of loop (B). Results are expressed as means ± standard errors of the means of results for each experimental condition ($n = 6$ to 10). **, $P < 0.01$ versus values for the control; +, $P < 0.05$, and +, $P < 0.01$ versus values for toxin A alone. CM, *S. bouhardii* conditioned medium; Tx A, toxin A; Ab, antibody; Sb, *S. bouhardii*.

and B were due to proteolysis of the toxin molecules, the nonspecific protease inhibitor α2-macroglobulin was incubated with purified protease and their proteolytic activity on tritiated toxins A and B was determined. Our results show that preincubation with α2-macroglobulin completely prevented digestion of both toxins by *S. bouhardii* protease (Table 1). In addition, preincubation of *S. bouhardii* conditioned medium with anti-*S. bouhardii* protease IgG inhibited digestion of both ³H-toxin A and ³H-toxin B by 40 and 63%, respectively ($P < 0.01$; Table 1).

Anti-*S. bouhardii* protease IgG prevents the action of *S. bouhardii* on the ability of ³H-toxin A and ³H-toxin B to bind to human colonic BBM. We previously reported that exposure of ³H-toxin A to *S. bouhardii* protease results in diminished ³H-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 *S. bouhardii* protease on toxin A and B binding to human colonic BBM receptors. As expected ³H-toxin A and ³H-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 ³H-toxin A or B with purified *S. bouhardii* protease significantly reduced ³H-toxin A and B bind-

TABLE 1. Anti-*S. boulardii* protease IgG inhibits *S. boulardii*-mediated proteolytic activity against purified *C. difficile* toxins A and B^a

Tested mixture	Toxin digestion (dpm/ml)
³ H-toxin A + buffer.....	3,200 ± 500
³ H-toxin A + <i>S. boulardii</i> protease	6,760 ± 810**
³ H-toxin A + <i>S. boulardii</i> protease + α2-macroglobulin.....	2,800 ± 350 ⁺⁺
³ H-toxin A + <i>S. boulardii</i> CM.....	6,690 ± 440**
³ H-toxin A + <i>S. boulardii</i> CM + anti-protease IgG.....	4,100 ± 550 ⁺⁺
³ H-toxin A + <i>S. boulardii</i> CM + control IgG.....	7,200 ± 780**
³ H-toxin B + buffer (control).....	2,750 ± 140
³ H-toxin B + <i>S. boulardii</i> protease.....	5,330 ± 200**
³ H-toxin B + <i>S. boulardii</i> protease + α2-macroglobulin.....	3,250 ± 490 ⁺⁺
³ H-toxin B + <i>S. boulardii</i> CM.....	5,590 ± 520**
³ H-toxin B + <i>S. boulardii</i> CM + anti-protease IgG.....	1,970 ± 90 ⁺⁺
³ H-toxin B + <i>S. boulardii</i> protease + control IgG.....	4,930 ± 210**

^a Results are the means ± standard errors of the means of results of four separate experiments, each with triplicate determinations. *S. boulardii* conditioned medium (CM; 0.1 mg/ml) or purified protease (0.1 μg/ml) was incubated (30 min at 37°C) with either tritiated toxin A or tritiated toxin B (0.1 μg containing 40,000 dpm). The reaction was stopped by adding TCA, and the mixture was placed on ice for 30 min and then centrifuged (700 × g for 30 min at 4°C). The radioactivity content of the supernatant was measured by scintillation counting. In similar experiments, the effect of anti-*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. **, *P* < 0.01 versus values for corresponding controls; ⁺⁺, *P* < 0.01 versus values for *S. boulardii* protease or *S. boulardii* conditioned medium alone.

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 radio-labeled 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 trans-epithelial 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 enterocyte 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

TABLE 2. Anti-*S. boulardii* protease IgG prevents *S. boulardii*-mediated inhibition of ³H-toxin A and ³H-toxin B binding to human colonic BBM^a

Tested mixture	dpm bound per 50 μg of BBM protein
³ H-toxin A + buffer (control).....	140 ± 40
BBM + ³ H-toxin A.....	8,730 ± 200**
BBM + ³ H-toxin A + <i>S. boulardii</i> CM.....	3,370 ± 350 ⁺⁺
BBM + ³ H-toxin A + <i>S. boulardii</i> CM + anti-protease IgG.....	7,750 ± 380**
BBM + ³ H-toxin A + <i>S. boulardii</i> CM + control IgG.....	3,500 ± 250 ⁺⁺
³ H-toxin B + buffer (control).....	220 ± 70
BBM + ³ H-toxin B.....	8,850 ± 310**
BBM + ³ H-toxin B + <i>S. boulardii</i> CM.....	3,880 ± 420 ⁺⁺
BBM + ³ H-toxin B + <i>S. boulardii</i> CM + anti-protease IgG.....	9,150 ± 390**
BBM + ³ H-toxin B + <i>S. boulardii</i> CM + control IgG.....	4,200 ± 240 ⁺⁺ **

^a 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.

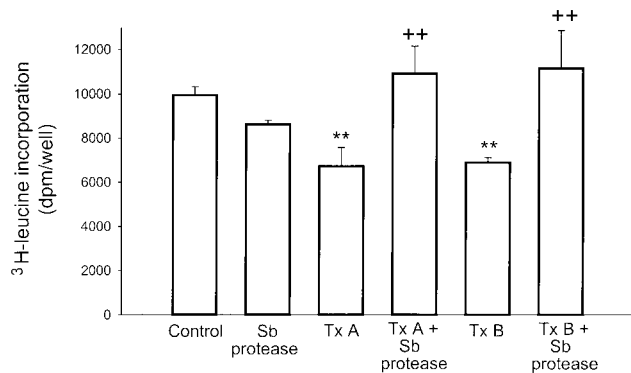


FIG. 3. *S. boulardii* protease prevents toxin A- and B-mediated inhibition of protein synthesis in colonic epithelial (HT-29) cells. Purified toxin A or B (1 μ g/ml) was incubated (1 h at 37°C) with either purified *S. boulardii* conditioned medium (100 μ g/ml) or buffer alone before addition to cultured HT-29 cells (10⁶ cells per well). After 4 h [³H]leucine (3 μ Ci/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 \pm 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*.

B-mediated effects in human colonocytes in vitro. We report that the 54-kDa *S. boulardii* protease, in addition to cleaving toxin A, possesses enzymatic activity against *C. difficile* toxin B (Fig. 1). Moreover, the *S. boulardii* protease diminishes the ability of toxins A and B to bind to human colonic BBM (Table 2) and inhibits the effects of both toxins on colonic epithelial cells and native human colonic mucosa in vitro (Fig. 3 and 4). This study is the first demonstration that the *S. boulardii* protease inhibits the action of both *C. difficile* toxins in human colon and that it may be relevant to the mechanism by which this nonpathogenic yeast exerts its beneficial effects in human *C. difficile* colitis. To our knowledge this is a unique mechanism of action for a biotherapeutic agent.

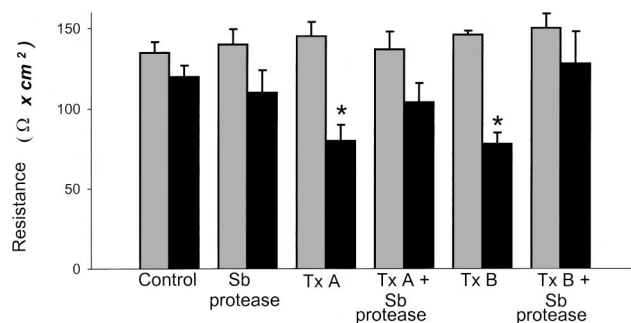


FIG. 4. *S. boulardii* protease prevents toxin A- and B-mediated reduction of colonic resistance. Human colonic mucosal sheets were placed in Ussing chambers and incubated (3.5 h at 37°C) with either buffer alone or buffer containing 32 nM toxin A, 3 nM toxin B, or purified *S. boulardii* protease (1 μ g/ml). Where indicated, toxin A or toxin B was preincubated (1 h at 37°C) with *S. boulardii* protease prior to placement in the Ussing chambers. Potential differences and short-circuit currents were recorded every 30 min to calculate changes in resistance (ohms per square centimeter). Results are expressed as means \pm standard errors of the means of results for each group. Resistance baseline values 10 min before application of toxins (light gray bars) and 3.5 h after exposure to toxins (dark gray bars) are shown. Six to eight chambers were tested for each experimental condition. *, $P < 0.01$ versus the 10-min values. Sb, *S. boulardii*; Tx, toxin.

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 radiolabeled 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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