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Saccharomyces boulardii Protease Inhibits *Clostridium difficile* Toxin A Effects in the Rat Ileum

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***Saccharomyces boulardii*, a nonpathogenic yeast, is effective in treating some patients with *Clostridium difficile* diarrhea and colitis. We have previously reported that *S. boulardii* inhibits rat ileal secretion in response to *C. difficile* toxin A possibly by releasing a protease that digests the intestinal receptor for this toxin (C. Pothoulakis, C. P. Kelly, M. A. Joshi, N. Gao, C. J. O'Keane, I. Castagliuolo, and J. T. LaMont, Gastroenterology 104: 1108–1115, 1993). The aim of this study was to purify and characterize this protease. *S. boulardii* protease was partially purified by gel filtration on Sephadex G-50 and octyl-Sepharose. The effect of *S. boulardii* protease on rat ileal secretion, epithelial permeability, and morphology in response to toxin A was examined in rat ileal loops in vivo. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified *S. boulardii* protease revealed a major band at 54 kDa. Pretreatment of rat ileal brush border (BB) membranes with partially purified protease reduced specific toxin A receptor binding (by 26%). Partially purified protease digested the toxin A molecule and significantly reduced its binding to BB membranes in vitro (by 42%). Preincubation of toxin A with *S. boulardii* protease inhibited ileal secretion (46% inhibition, $P < 0.01$), mannitol permeability (74% inhibition, $P < 0.01$), and histologic damage caused by toxin A. Thus, *S. boulardii* protease inhibits the intestinal effects of *C. difficile* toxin A by proteolysis of the toxin and inhibition of toxin A binding to its BB receptor. Our results may be relevant to the mechanism by which *S. boulardii* exerts its protective effects in *C. difficile* infection in humans.**

Clostridium difficile causes antibiotic-associated colitis and diarrhea in animals and humans (1, 2) and is now recognized as one of the most common nosocomial infections (21). Inflammatory colitis and diarrhea associated with *C. difficile* are caused by release of two protein exotoxins, toxin A and toxin B (15, 31). Although the intestinal mechanism of action of toxin A is not completely understood, binding of the toxin to its brush border receptor appears to be required for expression of enterotoxic effects (10, 12, 38). Injection of toxin A, a 308-kDa protein (11), into rodent intestine causes fluid secretion, increased mucosal permeability and mucosal damage, and release of inflammatory mediators (24, 34, 36, 37).

Saccharomyces boulardii, a nonpathogenic yeast, is effective both in the prevention and in the treatment of antibiotic-associated diarrhea and colitis in humans. Results from two double-blind controlled prospective studies indicated that the frequency of antibiotic-related diarrhea was significantly reduced in patients receiving oral *S. boulardii* compared with placebo (22, 32). McFarland et al. (23) recently reported that patients with acute relapse episodes of *C. difficile* disease receiving *S. boulardii* in combination with vancomycin or metronidazole experienced fewer recurrences compared with patients who received placebo. Buts et al. (5) reported rapid symptomatic and bacteriologic recovery in 16 of 19 children treated with *S. boulardii* for acute *C. difficile* infection. *S. boulardii* treatment also protected animals from the effects of either toxigenic *C. difficile* or purified toxin A administration (8, 9, 13, 19, 26, 33).

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S. boulardii may protect against *C. difficile* infection in mice or hamsters by either inhibiting production of toxins by the bacteria or acting directly on the *C. difficile* toxin molecules (8). However, incubation of *C. difficile* toxins with *S. boulardii* whole yeast or culture-conditioned medium failed to inhibit toxin A-mediated cell rounding in vitro (26), suggesting no effect of the yeast against the toxin molecule. Other proposed mechanisms for the antidiarrheal effects of *S. boulardii* include stimulation of intestinal disaccharidase activity (3) and enhanced mucosal immune response (4). An earlier study from our laboratory indicated that *S. boulardii* inhibits the enterotoxic effects of *C. difficile* toxin A in a rat ileal loop model of toxin A enteritis and that a yeast protease may be responsible for these inhibitory effects (26). The current study was undertaken to purify and characterize the *S. boulardii* protease and to examine in detail its mechanism of action in toxin A-mediated enteritis in rat ileal loops in vivo.

MATERIALS AND METHODS

Materials. Male Wistar rats (200 to 250 g) were purchased from Charles River Breeding Laboratories (Wilmington, Mass.). Animals were subjected to fasting 16 h before the study but provided with water. Pentobarbital sodium for anesthesia was obtained from Abbott (North Chicago, Ill.). Sabouraud dextrose broth for cultures of *S. boulardii* was purchased from Difco (Detroit, Mich.). Rabbit lung R9ab fibroblasts were purchased from the American Type Culture Collection (Rockville, Md.). Protein concentrations were estimated by the bicinchoninic acid protein assay reagent kit obtained from Pierce Laboratories (Rockford, Ill.) using bovine serum albumin as standard protein solution. D-(*N*)-[1-^{3}H] mannitol (30 Ci/mmol) was obtained from New England Nuclear (Boston, Mass.). The Bolton-Hunter reagent (*N*-succinimidyl [2,3-^{3}H]propionate; 80 Ci/mmol) was purchased from Amersham International (Amersham, England). For enzymatic treatment of [^{3}H]toxin A, protease from *Streptomyces griseus* type VI, trypsin from bovine pancreas, α-chymotrypsin from bovine pancreas, and carboxypeptidase B from porcine pancreas were obtained from Sigma Diagnostics (St. Louis, Mo.). All protease inhibitors used in this study were purchased from

Sigma, except dithiothreitol, which was obtained from Gibco BRL (Gaithersburg, Md.).

Methods. (i) *S. boulardii*. Lyophilized *S. boulardii* was provided by Biocodex Laboratories (Montrouge, France). *S. boulardii* powder was reconstituted to a final concentration of 0.1 mg/ml in Sabouraud dextrose broth and cultured for 48 h at 37°C. *S. boulardii* culture was then centrifuged (1,000 × *g* for 10 min at 4°C), and conditioned medium was aspirated and filtered through a 0.2-μm-pore-size membrane.

(ii) *Saccharomyces cerevisiae*. Protease-producing *S. cerevisiae* lyophilized cultures (ATCC 32167) were obtained from the American Type Culture Collection and cultured at 24°C for 48 h in 10 ml (50 million cells per ml) of YM broth (Difco). Conditioned medium was then collected, centrifuged, and filtered as described above.

(iii) Toxin purification and radiolabelling. Toxin A was purified from filtered supernatants of VPI strain 10463 and subsequently radiolabelled with the Bolton-Hunter reagent (Amersham) as previously described (12, 27). Enterotoxic activity of unlabelled and radiolabelled toxin A preparations was tested against rat ileal loops (24–26), and cytotoxic activity was tested against rabbit lung R9ab fibroblasts (27). Purity of unlabelled and labelled toxin A was assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (16). Purified toxin A preparations used in this study contained a single protein band in SDS-PAGE at ~300 kDa.

(iv) Protease assay. Proteolytic activity in *S. boulardii* conditioned medium was assayed as described by Roth et al. (30) by using the release of soluble radioactive fragments from the substrate [*methyl*-^{14}C]methemoglobin. *S. boulardii* conditioned medium or protease-containing fractions obtained from various purification steps (see below) were incubated with 5 μl (1 μg) of substrate (50,000 dpm) in 1 ml of 0.2 M acetate buffer (pH 3.8) at 37°C in a shaking water bath. After 20 min, the reaction was terminated by cooling the samples on ice and adding 0.2 ml of 50% trichloroacetic acid. The reaction mixture was then centrifuged (2,000 rpm for 30 min, 4°C) (Beckman J-25; Beckman, Palo Alto, Calif.), 0.25 ml of the supernatant containing small methemoglobin peptides was added to 5 ml of scintillation fluid, and radioactivity was measured by scintillation counting. Our results indicated that *S. boulardii* conditioned medium digested [^{14}C]methemoglobin in a dose-dependent manner in that addition of 0.5, 1.0, 10, and 100 μg and 1 and 2 mg of *S. boulardii* conditioned medium per ml (n = 4 per dose) increased the level of radioactivity in the assay supernatant by 3.3-, 6.6-, 9.0-, 17.6-, 25.2-, and 28.0-fold, respectively, compared with addition of buffer (control, $n = 12$, $P < 0.01$ for all doses). No further increase in proteolytic activity was noted by addition of 5 mg of *S. boulardii* conditioned medium per ml (n = 4). In addition, heat-inactivated (125°C for 15 min) *S. boulardii* conditioned medium (500 μg/ml) had no proteolytic activity. Results were expressed as units of protease activity per milligram of *S. boulardii* protein. One unit of *S. boulardii* protease was calculated as the amount that released into the supernatant 40% of the total substrate radioactivity (disintegrations per minute) after 20 min of incubation at 37°C.

(v) Effect of *S. boulardii* growth on protease release. *S. boulardii* was reconstituted in Sabouraud dextrose broth as described above and incubated at 37°C in a shaking water bath. One-milliliter culture aliquots were collected at different time points (1, 2, 5, 10, 20, 30, and 40 h), and the cell number was determined by counting the number of yeast cells with a hemocytometer. Proteolytic activity was estimated in conditioned medium by the [^{14}C]methemoglobin assay described above. Results from three such experiments indicated that yeast growth peaked at 20 h, after which time the cell number started to decrease, whereas proteolytic activity significantly increased sixfold after 30 h and peaked at 40 h (7.8-fold increase).

(vi) Effect of *S. boulardii* protease on the toxin A molecule in vitro. In another series of experiments, we examined the proteolytic effect of *S. boulardii* on [^{3}H]toxin A. [^{3}H]toxin A (0.1 μg, containing 40,000 dpm) was incubated with octyl-Sepharose-purified *S. boulardii* protease (0.1 μg/ml; see below) in 1 ml of 50 mM Tris buffer (pH 7.4) for 20 min at 37°C. The effects of 5 μg of either trypsin, protease from *Streptomyces griseus* type VI, carboxypeptidase, or chymotrypsin per ml were compared under the same experimental conditions. The reaction was stopped by 0.2 ml of trichloroacetic acid, and after centrifugation (2,000 rpm for 30 min at 4°C) (Beckman J-25), 0.25 ml of the supernatant was assayed for radioactivity.

(vii) Purification of the *S. boulardii* protease. All purification steps were carried out at 4°C. *S. boulardii* filtered conditioned medium from 48-h cultures was concentrated fivefold on an Amicon PM-50 filter (Gelman Scientific). Ten milliliters of concentrated conditioned medium was then loaded on a Sephadex G-50 column (1.8 by 95 cm) (Sigma), equilibrated with 50 mM Tris buffer (pH 5.5). The column was eluted with the same buffer at a flow rate of 5 ml/h. Five-milliliter fractions were collected and monitored for protein by A_{280} and for proteolytic activity in vitro against the [^{14}C]methemoglobin substrate. Selected fractions were also tested for inhibition of toxin A-mediated enterotoxicity in rat ileal loops. Fractions possessing proteolytic activity in vitro and inhibiting toxin A enterotoxicity in vivo were then pooled and dialyzed against 1 liter of 1 M ammonium sulfate in 10 mM Tris buffer (pH 7.4) for 18 h with two changes. This material was loaded on an octyl-Sepharose column (2.5 by 10 cm) (Sigma) equilibrated with 1 M ammonium sulfate in 10 mM Tris buffer (pH 7.4). The column was washed with the same buffer until the eluate showed A_{280} of 0, and proteins adsorbed to the column were eluted with a linear gradient of 1.0 to 0.0 M ammonium sulfate in 10 mM Tris buffer (pH 7.4). Five-milliliter fractions were collected and monitored for protein A_{280}. Protein-containing fractions were then dialyzed against 1 liter of 10 mM Tris buffer (pH 7.4) overnight and then assayed for proteolytic activity in vitro and for inhibition of toxin A-induced enterotoxicity in vivo. Our results showed that octyl-Sepharose-purified *S. boulardii* protease caused proteolysis of [^{14}C]methemoglobin in a dose-dependent fashion. Incubation of [^{14}C]methemoglobin with 0.005, 0.05, 0.1, 0.2, and 0.5 μg of purified protease per ml (n = 4 per dose) released 18.5, 41.4, 51.0, 60.4, and 70.7% of the total substrate radioactivity into the assay supernatant, respectively, whereas addition of buffer (control, n = 12) released only 6.4% of the total substrate radioactivity. No further increase in proteolytic activity was present by addition of 1 μg of purified protease per ml (n = 4).

(viii) pH dependency of *S. boulardii* protease activity. In these experiments, purified *S. boulardii* protease (1 U) was preincubated (22°C for 30 min) with a 0.2 M concentration of the following buffers: sodium phosphate (pH 2 and 3), sodium acetate (pH 3.8), potassium phosphate (pH 6 and 7), and Tris base (pH 8, 9, and 10) in a total volume of 1 ml. The pH of the various buffers was adjusted by adding either hydrochloric acid or sodium hydroxide. Protease activity was then estimated with the [^{14}C]methemoglobin method described above. Three such experiments were performed, each with duplicate determinations, for each datum point.

(ix) Effect of protease inhibitors on the proteolytic activity of *S. boulardii* protease. Purified *S. boulardii* protease (1 U in 1 ml of 0.2 M sodium acetate buffer, pH 3.8) was incubated (30 min at 22°C) with either buffer alone (control, n = 14) or a 1 mM concentration of the following protease inhibitors: phenylmethylsulfonyl fluoride, dithiothreitol, 2-mercaptoethanol, EDTA, iodoacetamide, leupeptin, diisopropyl fluorophosphate, dichloroisocoumarin, and phosphoramidon (n = 3 to 5 per group). The effect of 0.1 mg of either α_2-macroglobulin (n = 3) or captopril (n = 4) per ml was also tested under the same conditions. Protease activity against the [^{14}C]methemoglobin substrate was then measured, and results were expressed as percentage of control activity. In similar experiments, the effect of these protease inhibitors on the activity of purified *S. boulardii* protease (1 U) on [^{14}C]methemoglobin was also tested with a 0.2 M Tris base buffer at pH 7.4.

(x) PAGE. SDS gel electrophoresis was performed on 7.5% acrylamide separating slab gels (6 by 8 cm) with the discontinuous buffer system described by Laemmli (16). Samples of 20 to 40 μl were incubated in sample buffer containing 1.25% SDS and 5% 2-mercaptoethanol for 30 min and then heated at 100°C for 2 min, and electrophoresis was carried out at 50 mA for 3.5 h. Gels were fixed in a methanol-acetic acid-water (45:10:45) solution for 1 h, and protein bands were stained with Coomassie brilliant blue R-250. Migration of *S. boulardii* proteins was compared with migration of standard proteins (Bio-Rad Laboratories, Richmond, Calif.).

(xi) Effect of *S. boulardii* protease on toxin A-mediated intestinal effects. Rats were anesthetized by an intraperitoneal injection of pentobarbital sodium, and two 5-cm closed ileal loops were formed. Renal pedicles were tied to prevent renal excretion of [^{3}H]mannitol, and 10 μCi of [^{3}H]mannitol was injected intravenously. Each loop was then inoculated with either 5 μg of toxin A in 0.4 ml of 50 mM Tris buffer (pH 7.4) or 5 μg of toxin A which was preincubated for 5 or 15 min at room temperature with either Sephadex G-50-purified *S. boulardii* protease (100 μg) or octyl-Sepharose-purified protease (8 μg) or with 3.3 mg of filtered conditioned medium of either *S. boulardii* or *S. cerevisiae* cultures. In some experiments, ileal loops were first inoculated with octyl-Sepharose-purified protease (8 μg) for 15 min. Loops were then washed twice with 2 ml of 50 mM Tris buffer and then injected with 5 μg of toxin A. The abdomen was then closed in two layers, and animals were maintained under light anesthesia. After 4 h, the animals were sacrificed and the loops were removed. In some experiments, full-thickness sections of ileal loops were fixed in formalin and paraffin embedded, and sections were stained with hematoxylin and eosin and examined with a Nikon phase-contrast microscope (magnification, ×300; Nikon Inc., Garden City, N.J.). The histologic severity of enteritis was graded as previously described by us (6, 24, 25). Intestinal permeability was quantitated by blood-to-lumen excretion of [^{3}H]mannitol (6, 24, 26), and results were expressed as disintegrations per minute of [^{3}H]mannitol per centimeter of loop. Secretion of fluid was estimated as the weight/length ratio (milligrams per centimeter) of each loop as previously described (6, 24, 26).

The study was approved by the Boston University Medical Center and Beth Israel Hospital Institutional Animal Care and Use Committee.

(xii) Effect of *S. boulardii* protease on toxin A-mediated cell rounding. The effect of *S. boulardii* on toxin A-mediated cell rounding was evaluated with rabbit lung R9ab fibroblasts. Cells were grown in 250-ml Falcon tissue culture flasks (Becton Dickinson, Lincoln Park, N.J.) and seeded onto 96-well tissue culture plates (Costar, Cambridge, Mass.) as previously described for IMR-90 fibroblasts (26). Experiments were performed on a confluent monolayer of R9ab cells containing ~40,000 cells in 0.1 ml of medium. One-hundred-microliter aliquots from octyl-Sepharose-purified *S. boulardii* protease containing 8 μg of protein were incubated with 100 μl of 50 mM Tris (pH 7.4) containing various amounts (5 μg to 100 ng) of purified toxin A or buffer alone for 2 h at 22°C. Serial twofold dilutions were then made with 50 mM Tris buffer, and 100 μl of each dilution was added to R9ab cells in duplicate and incubated for 24 h at 37°C. Cell monolayers were then observed after 24 h for cell rounding, expressed as percentage of 200 cells.

TABLE 1. Purification of *S. boulardii* protease

Step	Total protein (mg)	Total activity (units)[a]	Sp act (units/mg)	Purification (fold)
S. boulardii medium	3,300	66,000	20	1
Sephadex G-50	48	48,000	1,000	50
Octyl-Sepharose	0.04	284	7,150	357

[a] One unit of *S. boulardii* protease was calculated as the amount that released 40% of the total substrate radioactivity (disintegrations per minute) into the supernatant after 20 min of incubation at 37°C.

(xiii) Effect of *S. boulardii* protease on the toxin A molecule. [^{3}H]toxin A (0.8 μg in 40 μl of buffer containing 160,000 dpm) was incubated with 8 μg of octyl-Sepharose-purified protease in a final volume of 0.5 ml of 10-mmol/liter Tris buffer or buffer alone (control) in a shaking water bath at 37°C. After 5 to 30 min, tubes were placed on ice and concentrated by freeze-drying to a final volume of ~10 μl. Equal volumes of Laemmli sample buffer containing 1.5% SDS and 2.5% 2-mercaptoethanol (final concentration) were added, and 15-μl aliquots of each sample were subjected to SDS-PAGE as described above. Gels were processed for autoradiography (17) with En^{3}Hance (New England Nuclear, Dupont) and developed after 2 weeks.

(xiv) Effect of *S. boulardii* protease on [^{3}H]toxin A binding to brush borders. Rat brush borders were purified from 10-cm-long ileal segments as described previously (26). Purified brush borders (30 μg per tube) were first preincubated at 37°C for 2 h with 20 μl of octyl-Sepharose-purified *S. boulardii* protease (10 μg per tube) or buffer alone (control) in 50-mmol/liter Tris buffer (pH 7.4) in a final volume of 0.2 ml. After incubation, brush borders were washed twice by centrifugation with 1 ml of Tris buffer (pH 7.4) to remove any residual enzyme. [^{3}H]toxin A binding in the presence or absence of a 100-fold excess of unlabelled toxin A was then measured at 22°C for 60 min as previously described (12, 26, 27). In some experiments, [^{3}H]toxin A (0.1 μg in 10 μl containing 40,000 dpm) was first preincubated for 2 h at 37°C with purified *S. boulardii* protease (10 μg in 10 μl) or buffer alone (control). This mixture was then added to purified rat ileal brush borders (30 μg) in a final volume of 0.2 ml, and [^{3}H]toxin A binding was measured as described above. Specific binding was calculated as described previously (12, 26, 27). Background radioactivity in tubes containing [^{3}H]toxin A but no membranes was subtracted.

RESULTS

Purification of *S. boulardii* protease. Purification of *S. boulardii* protease of more than 300-fold with a yield of 0.4% was obtained by sequential Sephadex G-50 and octyl-Sepharose chromatography (Table 1). The majority of *S. boulardii* proteolytic activity was eluted in the void volume of the Sephadex G-50 column (Fig. 1, peak A), whereas the major included peak (Fig. 1, peak B) did not contain any activity. We next tested the ability of *S. boulardii* proteins separated from the Sephadex G-50 column to inhibit toxin A-mediated enterotoxicity in vivo. Our prior studies indicated that 5 μg of toxin A stimulated a significant secretory response in the rat ileal loop (6, 24–26). Pooled *S. boulardii* fractions from the G-50 void volume (Fig. 1, peak A) significantly inhibited toxin A-mediated secretion (by 31.4%, $P < 0.01$) and [^{3}H]mannitol permeability (by 53.9%, $P < 0.01$), while pooled *S. boulardii* proteins from the included G-50 peak (Fig. 1, peak B) had no significant inhibitory effect (Table 2).

Void volume fractions from the Sephadex G-50 column containing proteolytic and in vivo toxin A inhibitory activity were pooled, dialyzed, and further purified by hydrophobic affinity chromatography on Octyl-Sepharose. After all nonadherent proteins were eluted, adherent proteins were eluted with a low ammonium sulfate concentration (Fig. 2). *S. boulardii* proteins contained in these fractions possessed proteolytic activity against [^{14}C]methemoglobin (Fig. 2). Moreover, 8 μg of protein from this fraction inhibited toxin A-induced rat ileal secretion (by 46%, $P < 0.01$) (Table 2) and [^{3}H]mannitol permeability (by 74.1%, $P < 0.01$) (Table 2).

We also tested the ability of the related protease-releasing yeast *S. cerevisiae* to inhibit toxin A-mediated enteritis. Preliminary experiments indicated that 1 μg of filtered conditioned medium per ml from 48-h cultures of *S. cerevisiae* ($n = 4$) showed similar proteolytic activity as 1 μg of *S. boulardii* conditioned medium per ml on the [^{14}C]methemoglobin assay (not shown). Preincubation of toxin A with filtered conditioned medium from 48-h cultures of *S. cerevisiae* did not significantly alter fluid secretion or [^{3}H]mannitol permeability caused by toxin A (Table 2). In contrast, preincubation of toxin A with the same amount of filtered *S. boulardii*-conditioned medium significantly inhibited both rat ileal fluid secretion and manni-

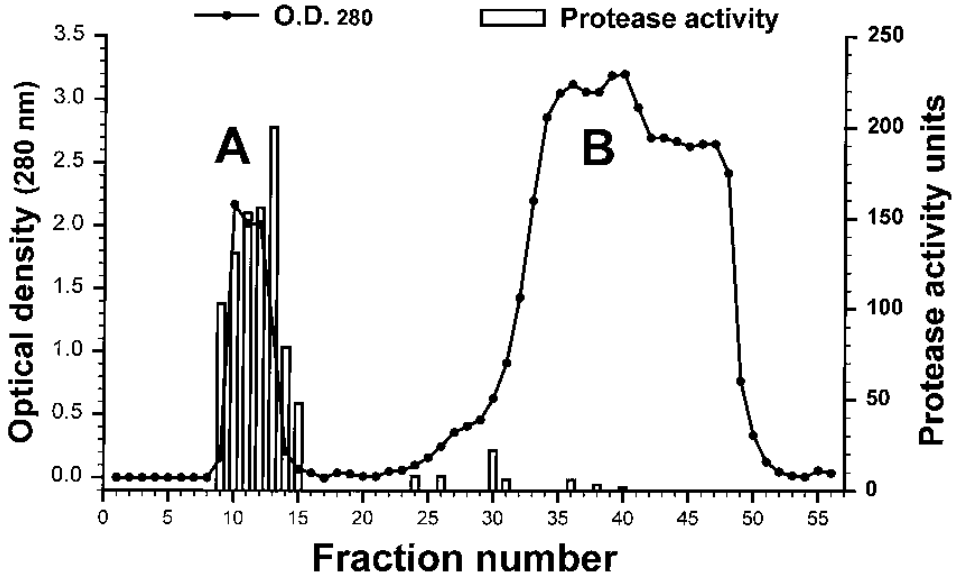

FIG. 1. Sephadex G-50 chromatography of *S. boulardii* conditioned medium. A sample of 10 ml containing 11 mg of protein per ml was loaded in 50 mM Tris buffer, pH 5.5, and eluted with the same buffer at 5 ml/h. Fractions of 5 ml were tested for protein (optical density at 280 nm) and protease activity against [^{14}C]methemoglobin as described in Materials and Methods. One unit of *S. boulardii* protease activity was calculated as the amount that digested 40% of total substrate radioactivity (disintegrations per minute) after 20 min of incubation at 37°C.

TABLE 2. Inhibition by *S. boulardii* protease of toxin A-mediated secretion and permeability in rat ileal loops[a]

Injection	Fluid secretion (mg/cm)	[^{3}H]mannitol permeability (dpm/cm of loop)
Buffer (n = 15)	109 ± 6	1,480 ± 290
Toxin A (n = 23)	379 ± 15**	47,710 ± 4,180**
Toxin A + Sepharose G-50		
Peak A (n = 10)	260 ± 31*,++	22,010 ± 5,770*,++
Peak B (n = 10)	314 ± 33**	31,830 ± 9,190**
Toxin A + octyl-Sepharose *S. boulardii* (n = 7)	208 ± 34**,++	11,470 ± 3,420**,++
Toxin A + *S. boulardii* conditioned medium (n = 6)	231 ± 26**,++	6,410 ± 1,950*,++
Toxin A + *S. cerevisiae* conditioned medium (n = 5)	318 ± 36**	29,840 ± 9,230*

[a] Ligated ileal loops were injected with 0.4 ml of 50-mmol/liter Tris buffer (pH 7.4) containing either 5 μg of toxin A or buffer alone or 5 μg of toxin A preincubated (22°C for 15 min) with proteins from the void volume (peak A, 100 μg) or included volume (peak B, 1.2 mg) of Sephadex G-50-purified *S. boulardii* conditioned medium (Fig. 1) or octyl-Sepharose-purified *S. boulardii* protease (8 μg) or *S. boulardii* or *S. cerevisiae* conditioned medium (3.3 mg). Loops were excised after 4 h, and secretion of fluid and [^{3}H]mannitol permeability were then measured as described in Materials and Methods. Results are expressed as means ± standard errors of the mean for each group. n represents the number of loops tested. *, $P < 0.05$ versus buffer; **, $P < 0.01$ versus buffer; $^{++}$, $P < 0.01$ versus toxin A.

tol permeability (Table 2), consistent with our previous results (26).

As shown in previous studies (6, 24), toxin A caused severe destruction of the rat villus epithelium, marked congestion and edema of the mucosa, and infiltration of the lamina propria with neutrophils (Fig. 3A). Preincubation of toxin A with octyl-Sepharose-purified *S. boulardii* protease reduced toxin A-mediated acute inflammation (Fig. 3B). Semiquantitative histology indicated that purified *S. boulardii* protease significantly inhibited villus damage caused by toxin A (Table 3). Although yeast protease reduced toxin A-mediated neutrophil infiltration and congestion and edema of the mucosa (Table 3), these results did not reach statistical significance.

Properties of octyl-Sepharose-purified *S. boulardii* protease. Protease-containing fractions from the Sephadex G-50 and octyl-Sepharose columns were pooled and electrophoresed on 7.5% polyacrylamide gels containing 1.25% SDS and 5% 2-mercaptoethanol. Whereas several protein bands were observed in the Sephadex G-50 protease fraction, a major band at 54 kDa was observed on the octyl-Sepharose-purified protease (Fig. 4). Two other minor protein bands of higher molecular weight were also observed (Fig. 4). To further characterize the protease, we tested the effects of several synthetic protease inhibitors on the activity of octyl-Sepharose-purified yeast protease at pH 3.8. Preincubation of protease with α_2-macroglobulin, a potent protease inhibitor, dramatically inhibited protease activity (by 84%, $P < 0.01$). Among several other inhibitors tested, only the serine-group-specific inhibitors diisopropyl fluorophosphate and dichloroisocoumarin (28) significantly inhibited protease activity (by 50%, $P < 0.01$, and 37%, $P < 0.05$, respectively), indicating that it is probably a serine protease. The reducing agents 2-mercaptoethanol and dithiothreitol did not significantly alter the protease activity. Iodoacetamide did not significantly reduce *S. boulardii* protease activity, indicating that sulfhydryl groups are not essential for activity. The metal-chelating agent EDTA also did not influence proteolytic activity, indicating that the yeast protease is not a typical metalloprotease. Similar results were obtained when the effect of the protease inhibitors was examined at pH 7.4 in that only α_2-macroglobulin, diisopropyl fluorophosphate, and dichloroisocoumarin significantly inhibited *S. boulardii* protease activity on the [^{14}C]methemoglobin substrate. A pH-enzymatic activity plot of the *S. boulardii* protease showed maximal protease activity at pH 4.0 (not shown). A second, much smaller peak of enzymatic activity was also observed at pH 8.0, indicating a possible heterogeneity in the purified *S. boulardii* protease fraction (not shown).

Digestion of toxin A by purified *S. boulardii* protease. The effect of the yeast protease on toxin A was then tested in three

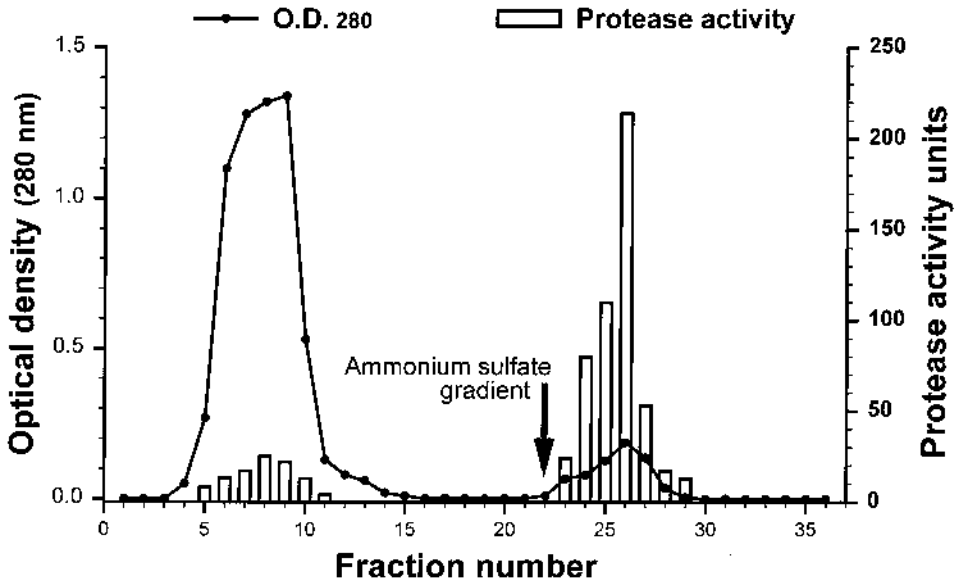

FIG. 2. *S. boulardii* protease purification on an octyl-Sepharose column. Ten milliliters of pooled protease-containing fractions from the Sepharose G-50 column containing 2.2 mg of protein per ml was dialyzed against 1 M ammonium sulfate in 10 mM Tris buffer (pH 7.4) and was loaded in the column equilibrated with the same buffer. The column was washed with buffer, and proteins absorbed to the column were eluted with a 1.0 to 0.0 M ammonium sulfate gradient. All fractions were monitored for protein (optical density at 280 nm), and selected fractions were monitored for protease activity against [^{14}C]methemoglobin.

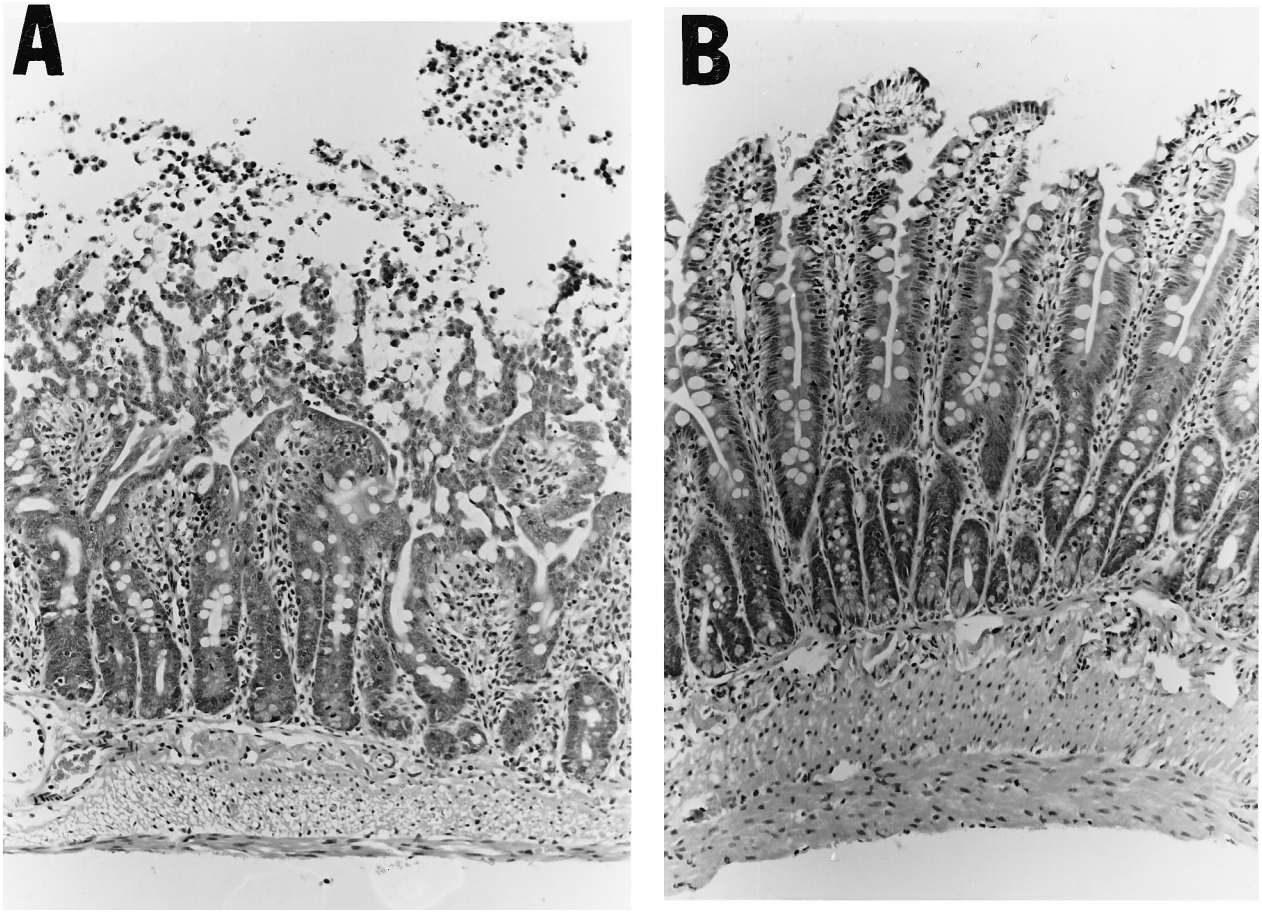

FIG. 3. Inhibition of toxin A-induced enteritis by *S. boulardii* protease. Rat ileal loops were injected with 5 μg of toxin A or toxin A preincubated with 8 μg of octyl-Sepharose-purified *S. boulardii* protease for 15 min at 22°C. After 4 h of exposure, rats were killed, full-thickness samples of ileal loops were fixed in formalin, and sections were stained with hematoxylin and eosin. (A) Toxin A-exposed loop showing villus necrosis and infiltration of the lamina propria with neutrophils. (B) Ileal loop exposed to toxin A preincubated with *S. boulardii* protease, showing complete prevention of the toxin A changes.

different sets of experiments. In the first, we compared the effects of purified protease and other enzymes on digestion of [^{3}H]toxin A as assessed by the trichloroacetic acid precipitation technique. Our results indicate that incubation of [^{3}H]toxin A with 0.1 μg of *S. boulardii* protease per ml ($n = 12$) resulted in significantly (2.85-fold) higher toxin proteolysis compared with incubation with buffer (control, $n = 7$, $P < 0.01$), whereas approximately 50-times-higher doses of *Streptomyces griseus* protease or chymotrypsin (5 μg/ml, $n = 7$ and 8, respectively) caused less toxin A proteolysis (1.37- and 1.33-fold, respectively) compared with control ($P < 0.05$ for both). Moreover, at a concentration of 5 μg/ml the proteases trypsin ($n = 8$) and carboxypeptidase ($n = 6$) did not degrade toxin A.

TABLE 3. Inhibition by *S. boulardii* protease of toxin A-induced enteritis[a]

Injection	Epithelial damage	Congestion and edema	Neutrophil infiltration
Control ($n = 21$)	0.3 ± 0.1	0.3 ± 0.1	0.40 ± 0.1
Toxin A ($n = 12$)	1.8 ± 0.1**	1.8 ± 0.3**	1.9 ± 0.2**
Toxin A + octyl-Sepharose *S. boulardii* ($n = 8$)	0.7 ± 0.3$^+$	1.5 ± 0.4**	1.5 ± 0.3**

[a] Ileal loops were injected with 0.4 ml of 50-mmol/liter Tris buffer (pH 7.4) containing either 5 μg of toxin A or buffer alone or an equal volume of octyl-Sepharose-purified *S. boulardii* protease (8 μg) preincubated (15 min at 22°C) with 5 μg of toxin A. Loops were excised after 4 h, and tissues were processed as described in the text. The histologic severity of enteritis was graded by a score of 0 to 3 for epithelial cell damage, congestion and edema of the mucosa, and neutrophil infiltration. Data are expressed as means ± standard errors of the mean for each group. n represents the number of loop samples examined. +, $P < 0.05$ versus toxin A; **, $P < 0.01$ versus control.

In the second approach, we monitored the time course of digestion of toxin A by the protease as assessed by autoradiography on SDS-acrylamide gels of [^{3}H]toxin A. Reduction of the intensity of the [^{3}H]toxin A band was evident 10 min after incubation with protease, and the band further diminished after 15 and 30 min incubation compared with the band produced by [^{3}H]toxin A exposed to buffer for 30 min (Fig. 5). The absence of additional bands in the protease-treated [^{3}H]toxin A samples indicates that the protease cleaves the toxin A molecule in multiple small peptide fragments not detected by autoradiography.

In the third approach, we examined the time course effect of preincubation of toxin A with yeast protease on toxin-induced rat ileal secretion. Preincubation of 5 μg of toxin A with 8 μg of octyl-Sepharose-purified *S. boulardii* protease showed progressive reduction of fluid secretion at 5 and 15 min (Fig. 6). These results, however, did not distinguish between an effect on the toxin A molecule and one on the toxin A receptor. In an attempt to separate these two mechanisms, we tested the effect

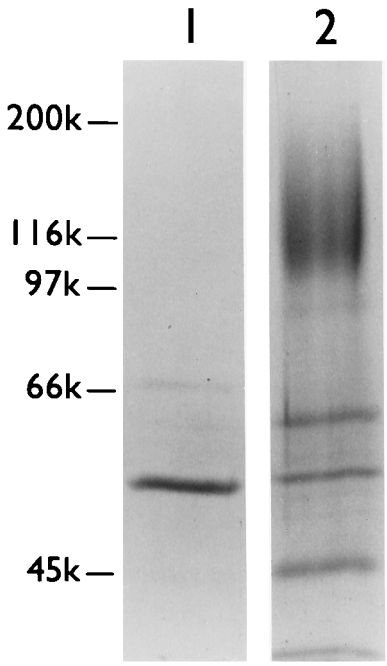

FIG. 4. SDS-PAGE of *S. boulardii* protease. Lane 1, 10 μg of octyl-Sepharose-purified *S. boulardii* protease. Lane 2, 10 μg of Sepharose G-50-purified *S. boulardii* protease. Samples were incubated in sample buffer containing 1.25% SDS and 5% 2-mercaptoethanol for 30 min and heated at 100°C for 2 min, and electrophoresis was carried out at 50 mA for 3.5 h. Gels were stained with Coomassie blue. Left, molecular weight standards: myosin (200k, where k represents 1,000), β-galactosidase (116k), phosphorylase *b* (97k), bovine serum albumin (66k), and ovalbumin (45k).

of exposure of ileal mucosa to protease for 15 min, followed by extensive washing and then toxin A administration. Under these experimental conditions, protease also significantly inhibited toxin A-induced secretion of fluid (Fig. 6), suggesting an independent effect on the receptor.

Effect of *S. boulardii* protease on toxin receptor binding. We next examined the ability of yeast protease to interfere with [^{3}H]toxin A binding to its rat brush border receptor. As expected from the above results (Fig. 5), pretreatment of [^{3}H]toxin A with octyl-Sepharose-purified protease reduced [^{3}H]toxin A-specific binding to rat ileal brush border by 42% (Table 4). A less substantial (26%) inhibition of binding was observed when protease was first preincubated with rat brush borders before addition of [^{3}H]toxin A to the membranes (Table 4), indicating that the protease is also effective against brush borders alone, although at a lower level than against toxin A alone. Under the same conditions, heat-inactivated protease had no inhibitory effect on [^{3}H]toxin A binding (Table 4). These results indicate that protease may have an enzymatic effect against toxin A and its intestinal receptor.

We next tested the effect of protease pretreatment on toxin A-induced cell rounding of fibroblasts in vitro. Preincubation of purified toxin A (5 μg to 100 ng) with octyl-Sepharose-purified protease (8 μg) for up to 2 h at 22 or 37°C before addition to cell monolayers did not affect toxin A-mediated cell rounding on R9ab fibroblasts. Incubation of cell monolayers with *S. boulardii* protease (8 μg) alone had no rounding effect on cultured fibroblasts.

DISCUSSION

In a previous study (26), we observed that *S. boulardii* conditioned medium inhibited binding of [^{3}H]toxin A to its intestinal receptor and reduced its intestinal effects in rat ileum in vivo. We report here that the protective effect of *S. boulardii* conditioned medium in the rat ileal loop model is attributable largely to a yeast serine protease that hydrolyzes toxin A and inhibits binding of this toxin to its brush border glycoprotein receptor. Our results suggest that *S. boulardii* protease more completely digests toxin A than do the four other proteases tested (see Results) and at a 50-fold-lower concentration. Previous investigators have commented on the unusual resistance of toxin A to pancreatic trypsin (18, 35) and its sensitivity to chymotrypsin (18), in keeping with the results presented here. Furthermore, *S. boulardii* conditioned medium was more effective in inhibiting the enterotoxic effects of toxin A in rat ileum than conditioned medium from a protease-releasing strain of *S. cerevisiae* (Table 2), despite the fact that conditioned media from both *Saccharomyces* strains were equally effective in causing proteolysis of the [^{14}C]methemoglobin substrate (see Results). It is not surprising that *S. boulardii* showed inhibitory effects in toxin A-mediated enteritis and *S. cerevisiae* did not, since these two species have been shown to be different (20). However, additional strains of protease-releasing *S. cerevisiae* should be studied in this and other animal models of toxin A-mediated diarrhea before we draw major conclusions on the effects of *S. cerevisiae* on *C. difficile* and its toxins.

Results of this study may be relevant to the mechanism by which *S. boulardii* exerts its therapeutic effects in *C. difficile* infection. Riegler et al. recently showed that both toxins A and B cause mucosal damage and electrophysiologic changes when applied to human colonic mucosal strips in vitro and that toxin B is more potent than toxin A in inducing these responses (29). Since toxin A is released into the bowel lumen during clinical

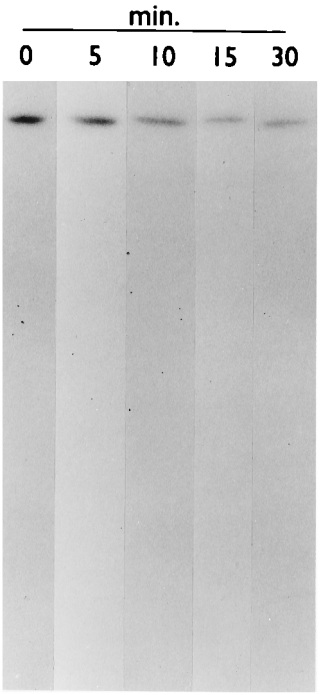

FIG. 5. Effect of *S. boulardii* protease on the toxin A molecule. [^{3}H]toxin A (0.8 μg containing 160,000 dpm) was incubated with 8 μg of octyl-Sepharose-purified *S. boulardii* protease at 37°C for the indicated times or with the same volume of 50 mM Tris buffer (pH 7.4) for 30 min (leftmost lane). Samples were then incubated with sample buffer containing 1.25% SDS and 5% 2-mercaptoethanol and subjected to SDS-PAGE as described in Materials and Methods. Gels were processed for autoradiography and developed after 2 weeks.

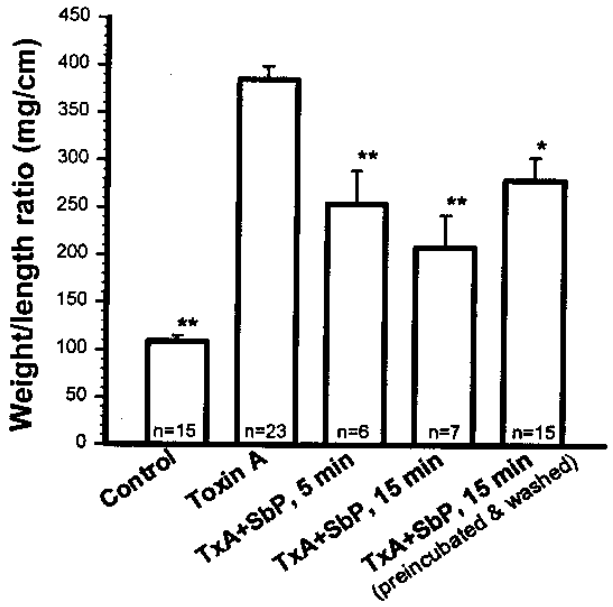

FIG. 6. Time course effect of *S. boulardii* protease on toxin A-mediated intestinal secretion. Ligated ileal loops were injected with 0.4 ml of 50-mmol/liter Tris buffer (pH 7.4) containing either 5 μg of toxin A or buffer alone or 5 μg of toxin A preincubated with 8 μg of octyl-Sepharose-purified *S. boulardii* protease (SbP) for 5 and 15 min at 22°C. In other experiments (far right bar), loops were first preincubated with 8 μg of purified *S. boulardii* protease for 15 min, then washed extensively with Tris buffer to remove excess protease, and then injected with 5 μg of toxin A. Loops were excised after 4 h, and secretion of fluid and [^{3}H]mannitol permeability were measured as described in Materials and Methods. Results are expressed as means ± standard errors of the mean for each group. *n* represents the number of loops tested. *, $P < 0.05$; **, $P < 0.01$ versus toxin A.

infection (15) and since the *S. boulardii* protease would also accumulate in the gut lumen following oral ingestion, we propose that gradual proteolysis of toxin A and probably toxin B would occur, thus reducing the effects of these potent toxins on the mucosa. In support of this contention, recent experiments in our laboratory indicate that preincubation of purified toxins A and B with *S. boulardii* conditioned medium inhibits release of interleukin-8 from human colonic adenocarcinoma cell lines in response to the toxins (6a). While we also observed inhibition of toxin A binding to its ileal brush border glycoprotein receptor (Table 4), we speculate that this may be less important than digestion of toxin A. Firstly, the yeast protease appears to be more active in vitro against the toxin than against purified brush borders (Table 4). In addition, we cannot exclude the possibility that in our binding experiments *S. boulardii* protease is not entirely removed by washing from brush border membranes. Membrane-bound *S. boulardii* protease could then possibly digest [^{3}H]toxin A, resulting in inhibition of toxin A binding. Secondly, we speculate that the colonic or ileal brush border receptor might be protected from the yeast protease by the mucus coat and unstirred water layer, which would protect diffusion of the protease.

Successful treatment of human (5) and experimental (7, 8, 14) *C. difficile* infection with *S. boulardii* results in decreased toxin levels in stools, an observation that could be partly explained by the results described here. Castex et al. (7) reported that *S. boulardii* pretreatment of mice subsequently challenged with *C. difficile* resulted in a 1,000-fold reduction of stool toxin titer but no significant change in *C. difficile* bacterial counts, a result consistent with toxin digestion by a yeast protease.

The ability of *S. boulardii* protease to reduce toxin A enterotoxicity but not fibroblast cell rounding is somewhat unexpected, especially in view of our finding that toxin A is hydrolyzed by the yeast protease (Fig. 5 and Table 4). One possible explanation for this discrepancy is that sufficient undegraded toxin remains after 2 h of incubation with the yeast protease to cause cell rounding. In fact, as shown in Fig. 5, after 30 min of incubation with yeast protease the [^{3}H]toxin A band at 308 kDa, although it is reduced in staining intensity, is still quite visible. Another possible explanation for these results is that rounding of fibroblasts may be mediated by toxin A fragments, while enterotoxic activity requires intact toxin.

TABLE 4. *S. boulardii* protease digests toxin A brush border receptor and [^{3}H]toxin A[a]

S. boulardii protease treatment	dpm bound per 30 μg of BB protein
Expt I	
Rat BB + buffer (control)	7,210 ± 590
Rat BB + *S. boulardii* protease	4,900 ± 200**
Rat BB + *S. boulardii* protease (inactivated)	8,550 ± 600
Expt II	
[^{3}H]toxin A + buffer (control)	6,130 ± 140
[^{3}H]toxin A + *S. boulardii* protease	3,350 ± 260**
[^{3}H]toxin A + *S. boulardii* protease (inactivated)	8,200 ± 80

[a] Results represent the means of three separate experiments per experimental approach, each with quadruplicate determinations. In experiment I, rat ileal brush border membranes (BB) (30 μg) were preincubated (2 h at 37°C) with buffer containing octyl-Sepharose-purified *S. boulardii* protease (10 μg per tube) or heat-inactivated (125°C for 15 min) *S. boulardii* protease or buffer alone in a final volume of 0.2 ml. After incubation, brush border membranes were washed and [^{3}H]toxin A-specific binding was measured as described in Materials and Methods. In experiment II, 10 μl of [^{3}H]toxin A (0.1 μg containing 40,000 dpm) was first preincubated (2 h at 37°C) with 10 μl of *S. boulardii* protease (10 μg); the mixture was then added to purified rat brush border membranes (30 μg/0.2 ml), and [^{3}H]toxin A-specific binding to rat brush border membranes was measured as described in Materials and Methods. Data are expressed as means ± standard errors of the mean per group. **, $P < 0.01$ versus respective controls.

In summary, our data provide a plausible mechanism for the therapeutic action of *S. boulardii* in patients infected with *C. difficile*. The yeast releases a serine protease that hydrolyzes toxin A and inhibits binding of the toxin to its intestinal receptor, thus diminishing the diarrhea and other intestinal effects of the toxin. In the experimental model used here, the yeast protease appears to account for most of the protective action present in conditioned yeast medium. Our results, however, do not exclude the other reported actions of *S. boulardii* (3, 4).

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