JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 5421–5426 5421
DOI:10.1021/jf904021b

Inhibition of Biological Activity of Staphylococcal Enterotoxin A (SEA) by Apple Juice and Apple Polyphenols

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The foodborne pathogen *Staphylococcus aureus* produces the virulent staphylococcal enterotoxin A (SEA), a single-chain protein that consists of 233 amino acid residues with a molecular weight of 27 078 Da. SEA is a superantigen that is reported to contribute to animal (mastitis) and human (emesis, diarrhea, atopic dermatitis, arthritis, and toxic shock) syndromes. Changes of the native structural integrity may inactivate the toxin by preventing molecular interaction with cell membrane receptor sites of their host cells. In the present study, we evaluated the ability of one commercial and two freshly prepared apple juices and a commercial apple polyphenol preparation (Apple Poly) to inhibit the biological activity of SEA. Dilutions of freshly prepared apple juices and Apple Poly inhibited the biological activity of SEA without any significant cytotoxic effect on the spleen cells. Additional studies with antibody-coated immunomagnetic beads bearing specific antibodies against the toxin revealed that SEA added to apple juice appears to be largely irreversibly bound to the juice constituents. The results suggest that food-compatible and safe anti-toxin phenolic compounds can be used to inactivate SEA *in vitro* and possibly also *in vivo*, even after induction of T-cell proliferation by long-term exposure to SEA. The significance of the results for microbial food safety and human health is discussed.

KEYWORDS: Staphylococcus enterotoxin A; apple juice; apple polyphenols; cell assays; inhibition; immunomagnetic beads; food safety

INTRODUCTION

Staphylococcus aureus is a major bacterial pathogen reported to cause clinical infections and contamination of a broad variety of foods, causing 185 000 cases of foodborne illnesses in the United States each year (*1*, *2*). Incidences and outbreaks associated with the consumption of food contaminated with *S. aureus* also occurred in other countries, including Austria (*3*), Italy (*4*), Japan (*5*), Portugal (*6*), and South Korea (*7*, *8*). It has been estimated that staphylococcal enterotoxin A (SEA) is associated with 78% of staphylococcal outbreaks (*9*).

S. aureus produces a group of 21 enterotoxins, many of which are heat-resistant in foods (*4*, *10*). Heat used to eliminate the pathogenic bacteria may not eliminate toxins already formed (*11*, *12*). Staphylococcal food poisoning is due to the absorption from the digestive tract into the circulation of the enterotoxins preformed in food (*13*).

Staphylococcus enterotoxins (SEs) have two separate biological activities: they cause gastroenteritis in the gastrointestinal tract and act as a superantigen on the immune system, contributing to the causes of mastitis in cattle (*14*) and emesis (*15*), rheumatoid arthritis (*16*), toxic shock syndrome (*17*), and atopic dermatitis (*18*) in humans. Previous research has shown that emetic activities and superantigenic activities of SEs are related (*15*, *19*).

Functional superantigens, such as SEA, are reported to exert their effects by binding to the α-helical regions of the major histocompatibility complex (MHC) class II molecules outside the peptide binding groove of the antigen-presenting cells (APCs) and also to the variable region (Vss) on T-cell receptors. The toxin then forms a bridge between T cells and APCs. This event then initiates the proliferation of a large number (∼20%) of T cells, which then induce the release of cytokines (*20*). At high concentrations, cytokines contribute to the causes of several known diseases. Because of their activity, SEs are included in the National Institute of Health/Center of Disease Control list of select agents that might be used as biological warfare agents (*21*−*23*). We selected spleen cells for this study because splenocytes are part of the immune system, containing a large number of APCs and T cells. They, therefore, provide a good target to study inhibition of SEs.

Because the toxin is present in contaminated foods and exerts adverse effects on the gastrointestinal tract, there is a need to find food-compatible safe conditions to inactivate it. Previous efforts to inhibit the toxin include the use of condensed tannins (*24*), high pressure and heat (*12*), radiation and pulsed electric fields (*25*), and electrolyzed water (*26*).

Previously, we devised an *in vitro* cell-based assay that distinguishes active from inactive SEA in milk (*27*), investigated the

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effect of heat used to pasteurize apple, grape, and orange juices on the formation of mutagens (*28*), and demonstrated that food-compatible plant compounds inactivated *Escherichia coli* O157:H7 and *Salmonella enterica* in apple juice (*29*) as well as both nonresistant and antibiotic-resistant strains of *S. aureus* in a pH 7 phosphate buffer (*30*). Apples are reported to decrease the incidence of symptoms associated with the toxin-induced atopic dermatitis in humans (*31*). The main objective of this study was to find out whether freshly prepared and commercial apple juices and a polyphenol-rich apple skin extract can inhibit the biological activity of SEA in cell assays.

MATERIALS AND METHODS

Materials. Two apple varieties and a bottle of commercially filtered pasteurized apple juice (S. Martinelli and Co., Watsonville, CA) were purchased from a local store (Safeway, Albany, CA). The apple varieties were Red Delicious and Golden Delicious, representing red- and golden-skinned varieties, respectively, grown in Washington. Preparation of the freshly prepared juices are described in detail elsewhere (*32*). The apple skin extract containing 82% polyphenols obtained was Apple Poly from Apple Poly LLC, Littleton, CO. SEA and anti-SEA antibodies were obtained from Toxin Technology (Sarasota, FL). The MultiTox-Glo Multiplex Cytotoxicity Assay kit (TB358) containing glycyl-phenylalanyl-aminofluorocoumarin (GF-AFC) was obtained from Promega (Madison, WI).

Isolation of Splenocyte Cells. Spleen cells from C57BL/6 female mice were prepared aseptically in Russ-10 cell culture medium and disrupted using a syringe and needle, made by combining 450 mL of RPMI 1640 medium without glutamine (Gibco, Carlsbad, CA), 50 mL of fetal bovine serum (Hyclone, Logan, UT), 0.25 mL of 100 mM β-mercaptoethanol (Sigma), and 5 mL of the following: 200 mM glutamine (Gibco), antibiotic–antimycotic containing penicillin, streptomycin, and fungizone (Gibco), non-essential amino acid mix (Gibco), and sodium pyruvate (Gibco). Cells were centrifuged at 200*g* at 4 °C for 10 min. Red blood cells were then lysed by adding red cell lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA). Cells were again centrifuged and resuspended in Russ-10 medium. Viable cells were counted using trypan blue and a hemocytometer.

Activity Assay for SEA. Cells were placed in 96-well plates (1×10^6/mL, 0.2 mL) in Russ-10 medium and treated with various concentrations of SEA following incubation at 37 °C in a 5% CO_2 incubator. After incubation at various time points, cell proliferation was measured by adding bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU)-labeled DNA to each well 4 h before fixation as described by the instructions of the manufacturer (Calbiochem, San Diego, CA). Spectroscopic measurements were made at absorbances of 620 and 450 nm.

Trypan Blue Exclusion Cell Viability Assay. An aliquot (50 μL) of the cell suspension was stained with trypan blue (50 μL; 0.4% in 0.85% saline) at room temperature for 3 min. Live and dead cells were then counted under a light microscope using a hemacytometer. Viable cells remained unstained, while dead cells were stained purple violet.

Splenocyte Cell Cytotoxicity Assay. In a second measure of cell viability, GF-AFC substrate (50 μL) was added to the wells. After mixing and incubation for 30 min at 37 °C, this substrate enters intact cells. The live cell protease then cleaves GF-AFC, releasing AFC generating a fluorescent signal. The resulting fluorescence was measured by a fluorescence plate reader (excitation at 355 nm and emission at 523 nm).

Tetrazolium (MTT) Assay. The MTT assay that differentiates dead from living cells was adapted from the literature (*33–36*). Briefly, spleen cells were plated on 96-well plates at 1×10^4 cells in 100 μL of Russ-10 medium per well. Samples (either 5 μL of juice in 95 μL of media or 100 μL of spiked media) were added to each well and incubated for 48 h at 37 °C in a 5% CO_2 incubator. MTT was diluted in phosphate-buffered saline (PBS) to 2 mg/mL followed by the addition of the diluent (25 μL) to each well. Plates were then incubated at 37 °C for 4 h, and the medium was removed. Dimethyl sulfoxide (DMSO) (100 μL) was added to each well, and plates were read at 540 nm.

Preparation of SEA Immunomagnetic Beads. Tosylactivated Dynabeads M-280 (100 μL) (Invitrogen, Carlsbad, CA) were washed twice with 0.1 M sodium borate buffer (600 μL) at pH 9.5 and then diluted in the same buffer to 2×10^9 beads/mL. Purified anti-SEA antibody (30 μg) was added to 1×10^8 beads (50 μL). The antibody and beads were incubated for 24 h at 37 °C on a slow shaker to facilitate covalent binding. The coated beads were washed twice for 5 min at 4 °C with 1 mL of PBS at pH 7.4 containing 0.1% bovine serum albumin (BSA), then washed once more for 4 h at 37 °C with 0.2 M Tris-HCl at pH 8.5 containing 0.1% BSA, and washed again for 5 min at 4 °C with PBS at pH 7.4 containing 0.1% BSA. The beads were then resuspended in Tris-BSA buffer (50 μL).

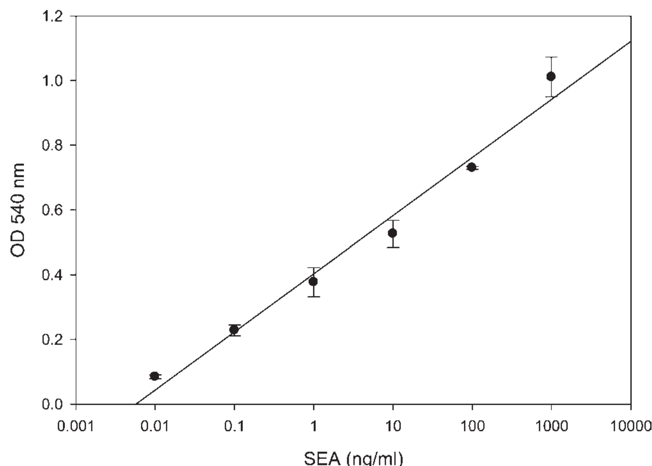

Figure 1. Linear relationship between the SEA concentration and DNA synthesis. To demonstrate stimulation of T cells, splenocyte cells were spiked with increasing concentrations of SEA. Newly synthesized DNA was measured at 540 nm after incubation for 48 h. Error bars ($n = 3$) represent standard errors.

Binding and Disassociation of SEA from Immunomagnetic Beads. The immunomagnetic beads (15 μL) were incubated with a tilting motion at 4 °C with apple juice (1 mL). After 24 h, the tube was placed on a magnet for 2 min to collect the beads. The beads were then washed twice with PBS at pH 7.4 containing BSA (0.1%). Toxin was eluted with 100 mM glycine HCl (7.5 μL, pH 2.5) and then neutralized with 2× Tris-buffered saline (TBS) (7.5 μL, pH 8.3).

Statistics. Statistical analysis was performed using SigmaStat 3.5 for Windows (Systat Software, San Jose, CA). Multiple comparisons of the spiked samples were made. One-way analysis of variance (ANOVA) was used to compare the control of unspiked apple juice with samples that contained increasing concentrations of SEA in the first experiment. The experiments were repeated at least 3 times. Results with $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Response of Splenocytes to SEA. To quantify the effect of superantigens on splenocyte cells, we measured the effect of SEA, a representative for the superantigen-released *S. aureus*. Various concentrations of SEA, ranging from 1000 to 0.01 ng/mL, were added to splenocytes. Cell proliferation was determined by a spectroscopic method that measures the incorporation of BrdU into newly synthesized DNA (*27*). Because rapid enzyme-linked immunosorbent assays (ELISAs) that have been developed for SEA do not distinguish between active and inactive toxins present in solution, we adopted our previously reported method to measure the effect of SEA on spleen cells based on the use of BrdU-labeled DNA (*37*). **Figure 1** illustrates the observed linear relationship between the SEA concentration ranging from 0.01 to 1000 ng/mL and the amount of newly synthesized DNA measured spectrophotometrically ($n = 3$; $R = 0.98$).

Apple Juices Inhibit Splenocyte Proliferation Induced by SEA. As already mentioned above, it has been previously reported that consumption of apples decreased and improved symptoms of human disease associated with superantigenic activity. Other

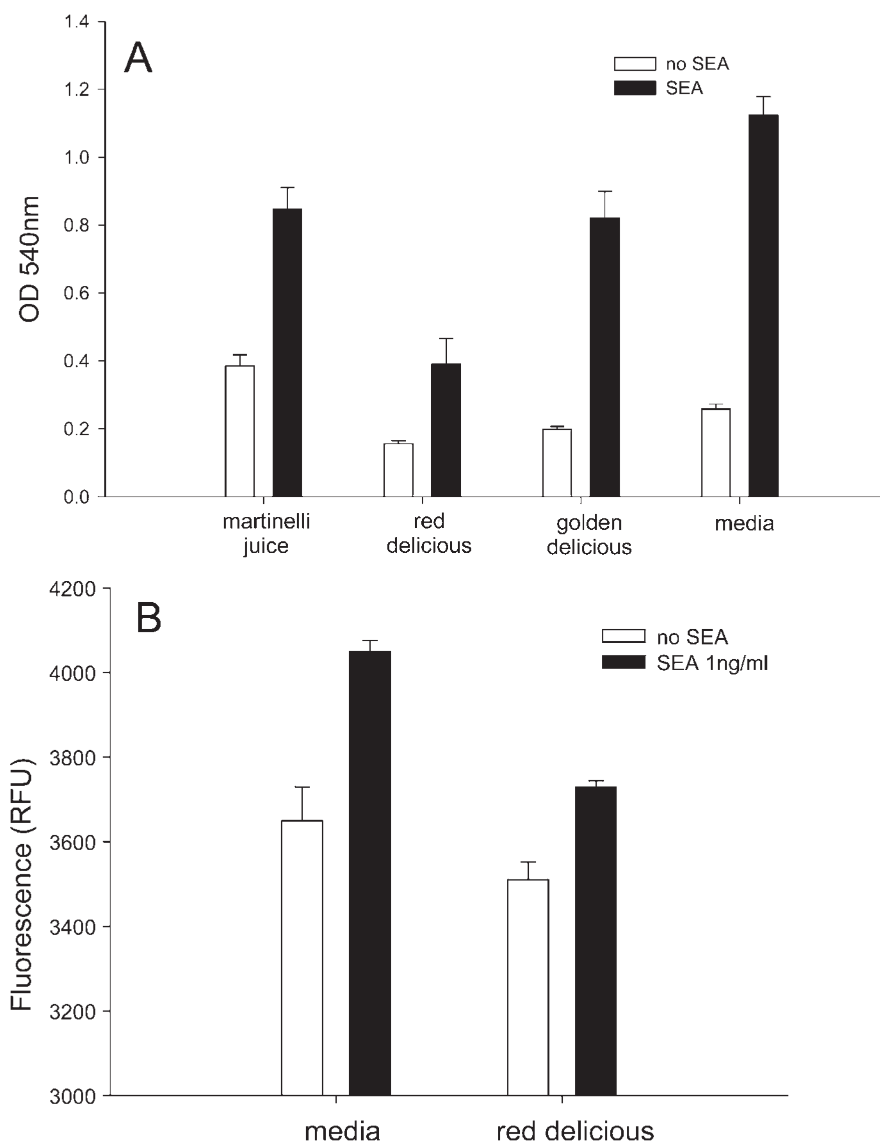

Figure 2. Effect of apple juice on splenocyte proliferation. Apple juice and media used as a control with or without SEA (1 ng/mL) were incubated for 48 h with splenocyte cells followed by determining newly synthesized DNA (A) by cleavage of the peptide GF-AFC and (B) by use of live splenocytes. Error bars ($n = 3$) represent standard errors.

studies reported that the polyphenolic compounds exhibit a strong affinity for proteins (*38−40*). It was therefore of interest to find out whether apple juice components can bind to and inhibit superantigenic activity of the SEA protein. To demonstrate whether this takes place, we incubated apple juice with or without SEA (1 ng/mL) in splenocytes for 48 h. T-cell proliferation was measured by two different methods: BrdU incorporation into newly synthesized DNA and cleavage of the peptide GF-AFC by a proteolysis enzyme only present in live and not dead splenocytes.

As shown in **Figure 2A**, the three types of apple juices tested inhibited SEA toxin activity as measured by the cell proliferation assay. Relative inhibitory activities of Red Delicious juice were greater than corresponding effects of Golden Delicious and Martinelli juices. A second experiment based on the GF-AFC assay that correlates directly with both the number and metabolic activity of live cells confirmed the inhibitory effect of Red Delicious apple juice (**Figure 2B**). There is no significant difference between unspiked media and Red Delicious juice, showing that Red Delicious juice does not have a cytotoxic effect on splenocyte cells. The two independent assays generated similar results. A one-way ANOVA statistical test showed that the 1:40 dilution of the apple juice that we used in all assays did not significantly affect cell viability ($p < 0.05$), as measured by the trypan blue exclusion assay, formazan product formation, and dead cell protease activity (data not shown).

Inhibitory Effects of Red Delicious Juice as a Function of the SEA Concentration. To determine whether the inhibitory activity of Red Delicious juice is limited to low concentrations of SEA, we also incubated for 3 h apple juice spiked with 1, 10, or 100 ng/mL SEA. These solutions were each added to splenocytes, and the toxin activity was then determined after 48 h. The inhibition of cell proliferation paralleled the SEA concentration (**Figure 3**).

Red Delicious Juice Inhibits T-Cell Proliferation Initiated by SEA. To determine whether treatment with Red Delicious apple juice will be effective after induction of T cells with long-term exposure to SEA, splenocyte cells were treated with three concentrations of SEA: 1, 10, or 100 ng/mL. After induction of cell proliferation for 24 or 48 h, apple juice without toxin was added to half of the treated solutions. The biological activities of these samples were then determined after incubation for an additional 24 h. The results show that the inhibition of SEA activity by the

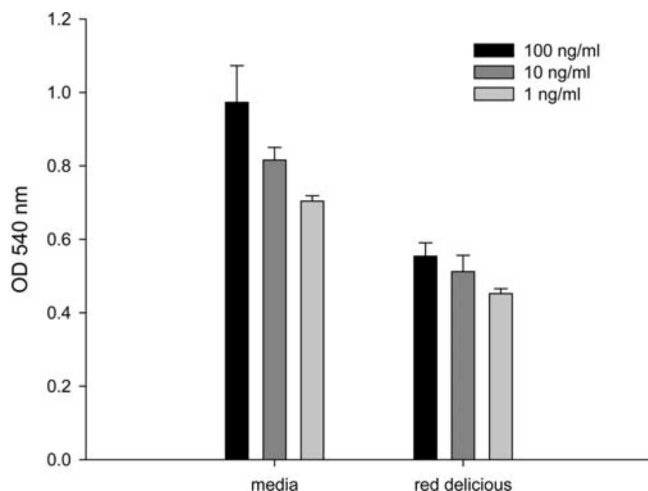

Figure 3. Red Delicious apple juice inhibits high SEA concentrations. Three concentrations of SEA were incubated for 48 h with splenocyte cells followed by determining newly synthesized DNA. Error bars (n = 3) represent standard errors.

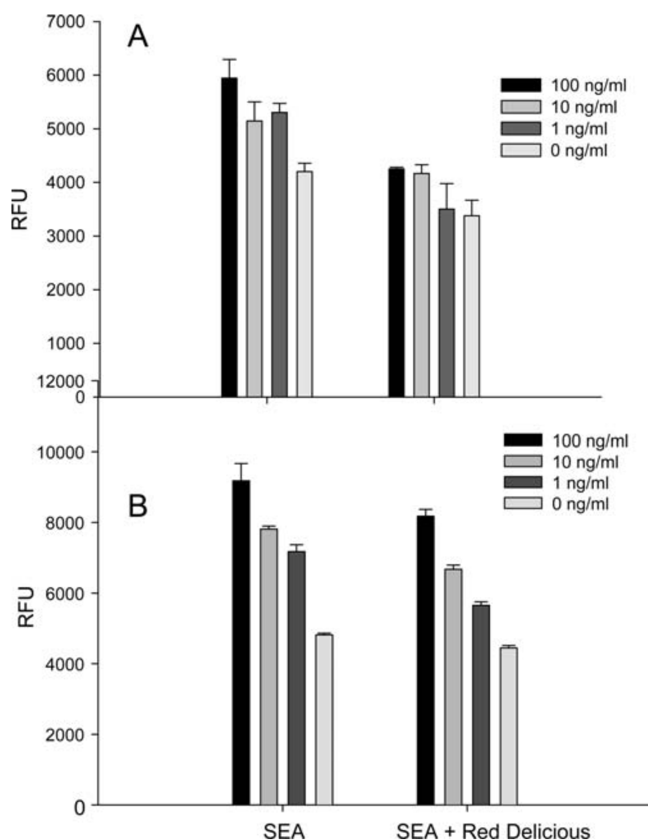

Figure 4. Red Delicious apple juice reduces the activity of SEA after 24 or 48 h of incubation. Different amounts of SEA were added to the splenocytes, which were then incubated for 24 or 48 h. This was followed by the addition of Red Delicious apple juice and the determination of biological activity by cleavage of GF-AFC, produced by the live splenocyte cells after (A) 48 h or (B) 72 h. Error bars (n = 3) represent standard errors.

added Red Delicious juice was statistically significant even after 24 or 48 h (panels **A** and **B** of **Figure 4**).

This observation suggests that Red Delicious juice has an inhibitory effect even after cell proliferation was initiated. We suggest that components of the juice disrupt the connection between APCs and T cells. Our results also imply that the mechanism by which consumed apple juice or apples may decrease the symptoms associated with atopic dermatitis is via inhibition of the proliferation of T cells and the release of cytokines. The postulated mechanism is visually illustrated in **Figure 5**.

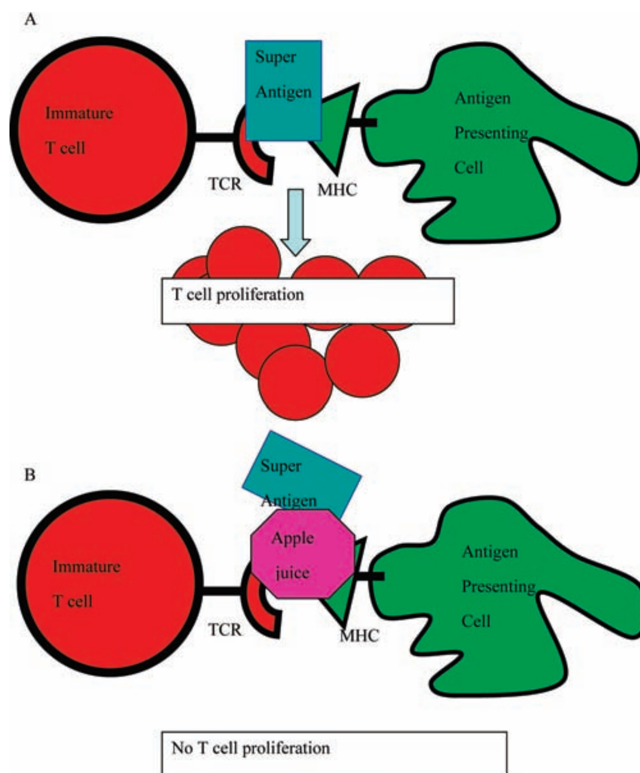

Figure 5. Schematic representation of cellular events that lead to the inhibition of SEA-induced cell proliferation by apple juice. The individual steps in this scheme involve the (A) formation of a bridge between APCs and T cells that results in induction of T-cell proliferation and (B) inhibition of T-cell proliferation by added pure apple juice that disrupts the connection between APCs and T cells. The net beneficial result of these events is the prevention of release and the consequent adverse effects induced by cytokines described in the Introduction. Abbreviations: MHC, major histocompatibility complex; TCR, T-cell receptor.

Reversibility of SEA Inhibition by Apple Juice. It is of interest to know whether SEA inhibition in apple juice is a permanent or reversible process. This phenomenon can be studied by determining if separating juice from SEA will result in the reactivation of the toxin. To demonstrate this possibility, we employed immunomagnetic beads coated with SEA-specific antibodies to extract SEA from juice. In these experiments, PBS, Red Delicious apple juice, and both filtered and unfiltered Martinelli apple juices were spiked with 1 ng/mL SEA and then incubated for 3 h. This was followed by the addition of 15 μL containing 30 $\times$ 10^6 immunomagnetic beads coated with anti-SEA antibodies and incubating the sample for an additional 16 h at 4 °C. After this mixing of the beads with the target toxin, they were washed multiple times to remove juice components. This was then followed by dissociating SEA from immunomagnetic beads with glycine-HCl (pH 2.5) and neutralization with 2$\times$ TBS (pH 8.3). The eluted toxin was added to splenocytes cells, and its activity was then measured as newly synthesized DNA.

Figure 6 shows that extraction and elution of SEA (1 ng/mL) from PBS gave a similar proliferative effect as observed by adding the same concentration of toxin to media. Specifically, when toxin was added directly to the cells, the absorbance value was 366.72 $\pm$ 45.7. This value is not significantly different from

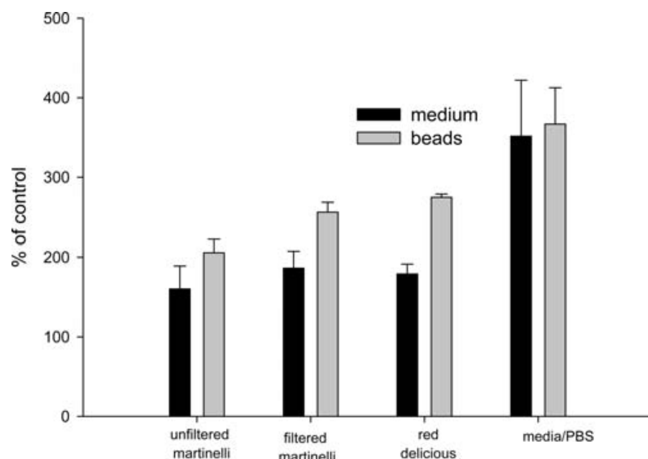

Figure 6. Extraction and elution of SEA from apple juice treated with immunomagnetic beads. Apple juices were spiked with SEA (1 ng/mL) and incubated for 16 h with immunomagnetic beads. The toxin was dissociated from the beads and incubated with spleen cells. This was followed by the determination of newly synthesized DNA. Error bars (n = 3) represent standard errors.

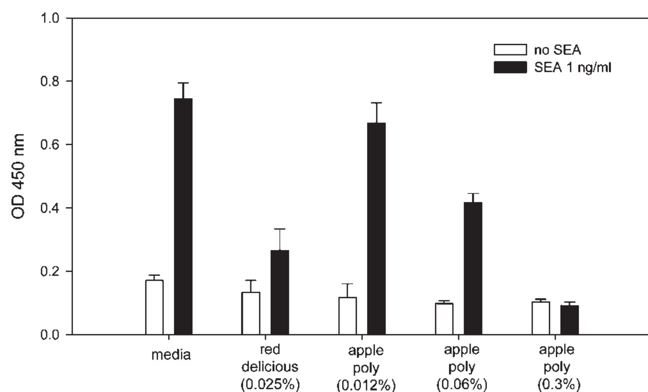

Figure 7. Comparison of inhibition of SEA by Red Delicious juice and Apple Poly. Splenocytes and SEA (1 ng/mL) were incubated for 48 h with Red Delicious juice or decreasing concentrations [0.3, 0.06, and 0.012% (w/v) in PBS] of Apple Poly, followed by determining newly synthesized DNA. Error bars (n = 3) represent standard errors.

351.7 ± 70.2, the value observed after the toxin was extracted and eluted from the apple juice. These results show that our method for toxin extraction and elution was very efficient.

Additional experiments showed that, when apple juice was added to toxin, it significantly reduced the biological activity of SEA. Even after extraction and washing, the apple ingredients were still bound to SEA and reduced its activity. Thus, the absorbance value for Red Delicious apple juice of 179.0 ± 12.2 after extraction and extensive washing changed to 274.69 ± 4.16. The corresponding change for Martinelli juice was from 160.22 ± 28.65 to 205.7 ± 16.55. These results show that that the apple juices but not the phosphate buffer used as a control significantly inhibited SEA activity, even after extensive washing designed to separate SEA from the juice components.

Inhibition of SEA by Apple Polyphenols (Apple Poly). A possible explanation for the observed differences in the extent of toxin inhibition between freshly prepared Red and Golden Delicious juices is that the Red Delicious apple variety is reported to contain higher levels of phenolic compounds than are present in Golden Delicious apples (*36−38*). To obtain additional evidence for this suggestion, we examined the ability of a concentrated commercial apple polyphenol to inhibit the superantigenic activity of SEA. The commercial Apple Poly product contains 82% polyphenols derived from skins of immature apples. Primary polyphenols in the extract include procyanidins, catechins, epicatechins, and chlorogenic acid (D. L. Kern, Apple Poly LLC, private communication). We do not know the distribution of individual phenolic compounds in the apple extract, only the total amount.

Specifically, splenocytes and SEA (1 ng/mL) were incubated for 48 h with decreasing concentrations [0.3, 0.06, and 0.012% (w/v) in PBS] of Apple Poly. At 0.3%, we observed a 25% decrease in splenocyte cell viability compared to control media. There was no loss in cell viability and no interference with the assay at the lower concentrations (0.06 and 0.012%), as determined by staining the intracellular components by trypan blue. Trypan blue is used to establish cell viability because it is excluded from the inside of healthy but not dead cells across the membrane.

As shown in **Figure 7**, diluted Apple Poly reduced the biological activity of SEA at concentrations that did not decrease cell viability. These observations indicate that (a) phenolic compounds in Apple Poly and apple juices appear to be responsible for toxin inhibition, possibly binding to multiple sites of the toxic protein, and (b) in studies of inhibition of toxins using cell assays, it is important to select inhibitor concentrations that inhibit only the toxin and not both the toxin and cells that participate in the assay. We separated these two effects using low concentrations (0.025% apple juices and 0.06 and 0.012% Apple Poly) of the toxin inhibitors.

In conclusion, the results of the present study show that the three apple juices evaluated inhibited the biological activity of SEA. A commercial apple skin extract that we previously found to facilitate heat inactivation of *E. coli* O157:H7 in cooked ground beef (*41*) also inhibited the biological activity of the toxin. Studies with magnetic beads coated with toxin-specific antibodies showed that the spiked toxin exposed to apple juice was largely not reactivated, even after washing the beads and removal of juice. Treatment with Red Delicious juice inhibited T-cell proliferation in response to SEA. The toxin then forms a bridge between APCs and T cells. It should be noted that, even 48 h after SEA initiates proliferation of T cells by the toxin, the proliferation can be inhibited by added apple juice (**Figure 4**). The inhibition of the spiked toxin was time-dependent and varied with the amount of toxin in the spiked juices. The described findings suggest that apple juices and polyphenol-rich apple skin extracts have the potential to counteract adverse effects in animals and humans induced by SEA and possibly also by the foodborne pathogen *S. aureus* that produces this virulent toxin. It would be of interest to find out whether or not the inhibited toxin in apple juice is reactivated in the digestive tracts of animals and humans and whether phenolic compounds present in other juices can concurrently inhibit the growth of *S. aureus* and other pathogens and the toxins produced by the pathogens.

ACKNOWLEDGMENT

We thank Carol E. Levin for preparing the fresh juices and Daphne Tamar and Sharon Abigail for their inspiration.

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Received for review March 1, 2010. Revised manuscript received March 29, 2010. Accepted April 9, 2010.