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Inactivation of Staphylococcal Enterotoxin-A with an Electrolyzed Anodic Solution

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Electrolyzed anodic NaCl solutions [EW(+)], prepared by the electrolysis of 0.1% NaCl, have been shown to instantly inactivate most pathogens that cause food-borne disease. Elimination of food-borne pathogens does not necessarily guarantee food safety because enterotoxins produced by pathogens may remain active. We have tested whether EW(+) can inactivate Staphylococcal enterotoxin A (SEA), one of the major enterotoxins responsible for food poisoning. Fixed quantities of SEA were mixed with increasing molar ratios of EW(+), and SEA was evaluated by reversed-phase passive latex agglutination (RPLA) test, immunoassay, native polyacrylamide gel electrophoresis (PAGE), and amino acid analysis after 30 min incubations. Exposure of 70 ng, or 2.6 pmol, of SEA in 25 μL of PBS to a 10-fold volume of EW(+), or ca. 64.6 $\times$ 10^3-fold molar excess of HOCl in EW(+), caused a loss of immuno-reactivity between SEA and a specific anti-SEA antibody. Native PAGE indicated that EW(+) caused fragmentation of SEA, and amino acid analysis indicated a loss in amino acid content, in particular Met, Tyr, Ile, Asn, and Asp. Staphylococcal enterotoxin-A excreted into culture broth was also inactivated by exposure to an excess molar ratio of EW(+). Thus, EW(+) may be a useful management tool to ensure food hygiene by food processing industries.

KEYWORDS: Electrolyzed NaCl solution; staphylococcal enterotoxin; food-borne disease; inactivation

INTRODUCTION

Ensuring the safety of food products is the first priority of the food industry. According to annual statistics provided by the Japanese Ministry of Health, Labor and Welfare, about 1,000 outbreaks of food-borne poisoning, involving 30,000 to 35,000 individuals, are reported each year in Japan. Because food-borne disease is caused mostly by infection with pathogenic microorganisms, three principles have been developed to aid in the prevention of food-borne disease: prevent microbial contamination, halt microbial growth, and protect against pathogens by disinfection.

Hazard analysis and critical control point (HACCP) is a procedure used to identify and correct potential sources of microbial contamination, and it is used by the food industry to ensure food safety. Novel technologies, such as ozone treatment (*1*, *2*) and electron-beam irradiation (*3*) have been proposed as useful tools in the HACCP process. Although useful, these technologies are expensive and are technically demanding.

Electrolyzed chlorous solutions, or electrolyzed oxidizing water [EW(+)] has attracted much recent attention as a low-cost, but high-performance, new technology of potential use by the food industry. The term "EW(+)" is used to describe an aqueous disinfectant produced by the electrolysis of a chlorine-containing solution under a low-voltage direct current (*4*, *5*). Venczel et al. (*6*) reported EW(+) as an effective disinfectant for *Cryptosporidium parvun* oocysts and *Clostridium perfringens* spores. The antimicrobial properties of EW(+) are reported to be due to the presence of hypochlorous acid, HOCl (*7*−*10*). Previous studies have reported the use of EW(+) to be practical and applicable for use in food-hygiene management; however, these studies have mainly concentrated on the inactivation of pathogenic microorganisms by EW(+). The inactivation of food-borne pathogens is not necessarily sufficient to prevent food-borne disease, because trace amounts of enterotoxin produced by the pathogenic bacteria may remain active after disinfection. Therefore, it would be useful to know whether EW(+) may eliminate or inactivate those enterotoxins.

Staphylococcal enterotoxins (SE) are proteins with molecular weights of 27 to 30 KDa. To date, eight SE have been reported (*11*−*19*); however, the most potent of these is Staphylococcal enterotoxin A (SEA) (*20*). A total mass of below 200 ng of SEA causes food-poisoning in humans, and concentrations of only 0.4 to 0.8 ng/mL leads to disease within 3−5 h after intake (*21*). SEA is stable against heat treatment; its toxicity remains even after a 30 min exposure to 100 °C (*22*). In addition, SEA is resistant to treatment with strong acid and alkali (*23*). Contamination of industrial food plants such as dairy plants (raw milk tank, or valve, etc.) with SEA, have caused large-scale

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outbreaks of food-borne disease; such a scenario occurred in a dairy processing plant around Osaka, Japan in 2000. To prevent poisoning caused by SEA, it is important to identify practical measures to eliminate or deactivate the toxin. In this paper, we describe the inactivation of SEA by EW(+) and provide data indicating that EW(+) might be useful as a preventive measure against food-borne diseases caused by SEA.

MATERIALS AND METHODS

Chemicals. Staphylococcal enterotoxin A (SEA) was purchased from Sigma-Aldrich (St. Louis, MO) and from Toxin Technology, Inc. (Sarasota, FL). A reversed-phase passive latex aggregation (RPLA) kit for the semiquantitative analysis of SEA was purchased from Denka Seiken Ltd. (Tokyo, Japan). Anti-Staphylococcal enterotoxin A and peroxidase-labeled affinity-purified antibody to rabbit IgG, used for quantification of SEA by ELISA, were purchased from Sigma-Aldrich (St. Louis, MO) and Kirkegaard & Perry Laboratories (Gaithersburg, MD), respectively. Chemical reagents and solvents used throughout this study were guaranteed reagent grade.

Generation of Electrolyzed Anode and Cathode NaCl Solutions. A diaphragm batch-scale electrolysis device (Superoxseed Labo JED-020, Aoi Electronics Co., Kannami, Shizuoka, Japan) equipped with a platinum chloride electrode was used. Electrolyses (12 min at room temperature) of dilute NaCl solutions were conducted with 0.1% NaCl dissolved in deionized water. Voltage was automatically controlled between 9 and 11 V of direct current. After electrolyses, strong acidic solutions (between pH 2.5 and pH 2.8) with available chlorine contents of about 36.3 ppm, that is equivalent to 0.67 mM HOCl and oxidation reduction potentials (ORP) of +1,180 mV, formed in the anode compartment. In contrast, strong alkaline solutions (pH 11.6 to pH 12.0) and ORP below −880 mV formed in the cathode compartment. For evaluating the disinfectant properties of the electrolyzed NaCl solution, both anode and cathode solutions were produced immediately before use. The available chlorine concentration was measured by iodometry and electrotitration using an available-chlorine meter (type HC-30, Central Kagaku Co. Inc., Tokyo, Japan).

Inactivation of Staphylococcal Enterotoxin A (SEA) with the Electrolyzed Solutions. *Organic-Free Medium.* A SEA (purchased from Sigma-Aldrich) used as stock suspension (2.8 μg/mL corresponding to ca. 100 nM) in phosphate-buffered saline (PBS, pH 7.4) was used as the source solution. First, 25 μL of the stock solution containing 70 ng SEA corresponding to 2.6 pmol was mixed with 0, 75, 125, or 250 μL of EW(+) that contained 0, 50, 84, and 168 nmol HOCl, respectively, or EW(−) that did not contain any reactive chlorine at all. SEA mixed with different volumes of EW(+) was incubated for 30 min at room temperature. After 30 min incubation, reactions were terminated by the addition of 100 μL of 1% mannitol, an OH radical scavenger (*24*), and then 400, 325, 275, or 150 μL of PBS were added to each incubated solution, respectively, so that a constant volume (400 μL) was achieved. From the 400 μL of incubated solution, aliquots (25 μL) of each reacted solution were analyzed by RPLA test according to the manufacturer's instructions. The RPLA test was repeated 5 times for each mixing ratio with EW(+). SEA (100 μg) used for analyses by RPLA and ELISA, for proteins by native PAGE, and for amino acid composition with HPLC, was similarly treated with EW(+) or EW(−) at a larger scale, and then provided for analyses.

Organic Medium, a Model Experiment on the Effect of EW(+) on the Inactivation of SEA in the Incubation Broth. A model experiment was run to examine whether SEA released into organic medium could be inactivated with EW(+). The suspension containing 90 ng corresponding to 3.5 pmol of SEA (purchased from Toxin Technology Inc., Sarasota, FL) in 25 μL of the brain−heart infusion broth was used as the model of SEA extracellularly excreted into incubation broth. SEA (90 ng) in 25 μL of brain−heart infusion broth was mixed with 3-, 5-, 10-, 25-, 50-, 75-, 100-, and 125-fold volumes, or 75, 125, 175, 250, 625, 1,250, 1,875, 2,500, or 3,125 μL, containing 50, 84, 168, 420, 840, 1,260, 1,680, and 2,100 nmol of HOCl, respectively, of EW(+) (converted from available chlorine 36.3 ppm; pH 2.31; ORP 1,158 mV) for 30 min. Then 100 μL of 10 mM thiourea, radical scavenger (*25*), was added to the incubated solution to terminate the reaction of EW(+) with SEA. From the incubated solution, 25 μL was taken to run the RPLA test. On the basis that the molecular weight of SEA is ca 28 kDa, and 75 μL of EW(+) of 36.5 ppm available chlorine contained 50 nmol of hypochlorous acid (HOCl), then 3-, 5-, 10-, 25-, 50-, 75-, 100-, and 125-fold volumes of EW(+) should correspond to 14×10^3-, 24×10^3-, 48×10^3-, 120×10^3-, 240×10^3-, 360×10^3-, 480×10^3-, and 600×10^3-fold molar excess of HOCl to SEA. As the control, SEA (90 ng) in brain−heart infusion broth (25 μL) was similarly mixed with 0.1% NaCl solution in place of EW(+), then subjected to the RPLA test. Results of the RPLA tests were assessed after a 24 h incubation at 37 °C, and then compared to that of the untreated SEA according to the table in the instruction manual.

Enzyme-Linked Immunosorbent Assay (ELISA). Because the RPLA test is a semiquantitative analytical technique, denaturation of SEA was also verified by ELISA using the method of Modi et al. (*26*) with slight modifications. Treatment of SEA with EW(+) was carried out by mixing 70 ng (2.6 pmol) SEA with 5- or 10-fold volume excess of EW(+), which corresponded to 32.3×10^3-, 64.6×10^3-fold molar excess of HOCl, or contained 84 or 168 nmol HOCl according to the procedure as described above. To each incubated solution, 100 μL of 10 mM thiourea was added to terminate the reaction of EW(+) with SEA. From the reacted solution, 150 μL was taken into multi-well microtiter plates (Falcon Microtest III flexible assay plate, Becton Dickinson and Co., Franklin Lakes, NJ) and incubated for 2 h (37 °C) to fix the SEA. Microtiter plates were then rinsed, and fixed SEA was blocked with 2% bovine serum albumin (BSA)/PBS for 30 min at room temperature. The BSA/PBS solution was removed, and the wells were washed 3× with 150 μL of 0.05% Tween-20 in PBS. Next, 150 μL of anti-SEA antibody (Sigma Chemical, St. Louis, MO) was added at a 20,000-fold dilution of the primary antibody, and the plates were incubated for 1 h at 37 °C. Unbound anti-SEA antibodies were removed, the wells were rinsed with Tween-20 in PBS, and 150 μL of 1,000-fold diluted peroxidase-labeled affinity-purified antibody to rabbit IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to each well and incubated for 1 h at 37 °C. The peroxidase-labeled antibody solution was removed, the wells were washed with Tween-20 in PBS, and 150 μL of a mixture of 0.03% 2,2′-azinodi-3-ethylbenzthiazoline sulfonate and 0.1% hydrogen peroxide was added and incubated for 1 h at 37 °C. Absorption in each well was read at 415 nm by a microplate reader (Corona Electric Co., type MTP-120, Tokyo, Japan). The same procedure was run with the SEA standard. The measurement was repeated with 5 samples.

Native PAGE Analysis. Native PAGE was used to examine the denaturation of SEA after exposure to EW(+). Native polyacrylamide gel electrophoresis was conducted according to Ledoux et al. (*27*) using 7.5% polyacrylamide gel and 25 mM Tris-190 mM glycine electrophoresis buffer (pH 8.3). Protein bands were visualized by Coomassie Brilliant Blue staining.

Amino Acid Composition. The amino acid composition of SEA after exposure to EW(+) was compared with that of untreated SEA. Control and EW(+)-treated SEA solutions were hydrolyzed with 6 N HCl containing 1% phenol at 110 °C for 24 h. Free amino acids released from SEA by hydrolysis were derivatized with phenylisothiocyanate (PTC), and then analyzed by HPLC using a reversed-phase PICO-TAG column (3.9 × 150 mm; Waters Associates) and monitoring UV absorption at 254 nm (*28*). The PTC−amino acid derivatives were eluted with by a programmed gradient of solution A (0.14 M sodium acetate containing a 50:3 ratio [v/v] of 0.2% v/v triethylamine, pH 6.8: acetonitrile) and solution B (60% acetonitrile). HPLC analysis was run three times, and the data were expressed as the mean for 3 measurements.

RESULTS AND DISCUSSION

Inactivation of Staphylococcal Enterotoxin A (SEA) with Electrolyzed NaCl Solution. *RPLA Test.* The inactivation of SEA by EW(+) or EW(−) was greatly affected by the volume (or molar excess) used. In the organic-free model, exposing SEA to an equal molar ratio of EW(+) did not have a measurable effect on SEA. However, exposure to a 3-fold volume excess

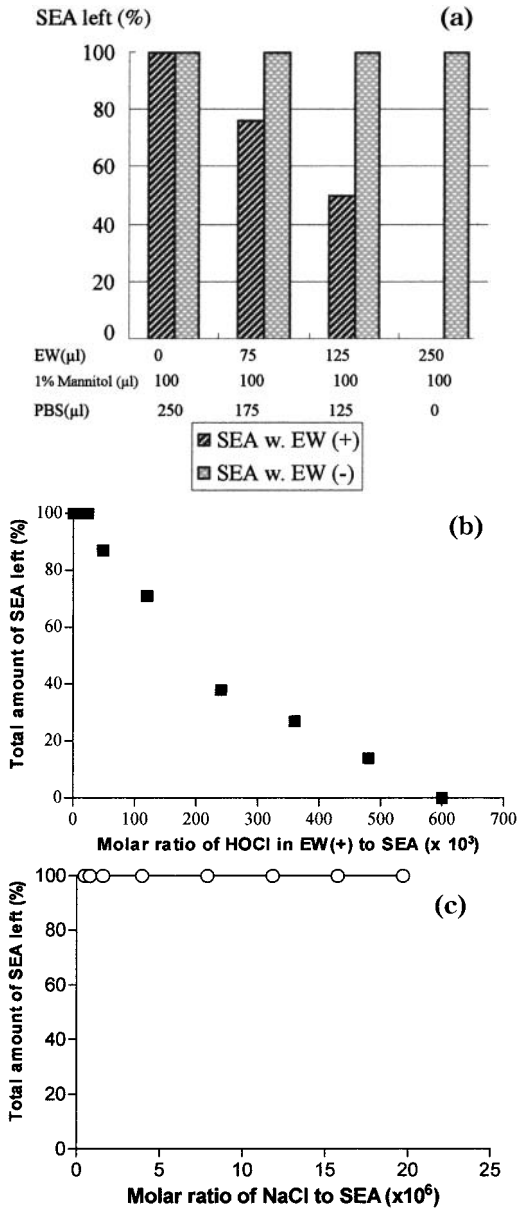

Figure 1. RPLA determination of the denaturation of Staphylococcal enterotoxin A after treatment with EW(+) and EW(–). (a) SEA remaining after exposure to increasing volume excess ratios of electrolyzed anodic [EW(+)] and molar ratio of HOCl in EW(+) or cathodic [EW(–)] solutions in organic-free conditions. SEA (70 ng) in 25 μL of PBS was exposed to 0, 75, 125, and 250 μL of EW(+) or EW(–) solution for 30 min, then 100 μL of 1% mannitol was added to each to terminate the reaction, followed by PBS to adjust the volume. SEA was then quantified by RPLA as described in the Materials and Methods section. The ordinate represents % amount of anti-SEA reactive SEA left, and the abscissa represents added EW solution (upper row), added 1% mannitol (middle row), and added PBS to adjust the final volume 350 μL. See text for full experimental details. The data are averages from 3 separate measurements. (b) SEA remaining after exposure to increasing molar ratios of EW(+) in nutrient broth. SEA was measured by RPLA as described in the Materials and Methods section. The ordinate represents % amount of anti-SEA reactive SEA left and the abscissa represents molar ratio of HOCl in EW(+) to SEA. (c) Remaining SEA % after the exposure of SEA to NaCl of different molar ratio. The ordinate represents % amounts of anti-SEA reactive SEA left and the abscissa represent molar ratio of NaCl to SEA. Full experimental details are given in the text. The data are averages from 3 separate measurements.

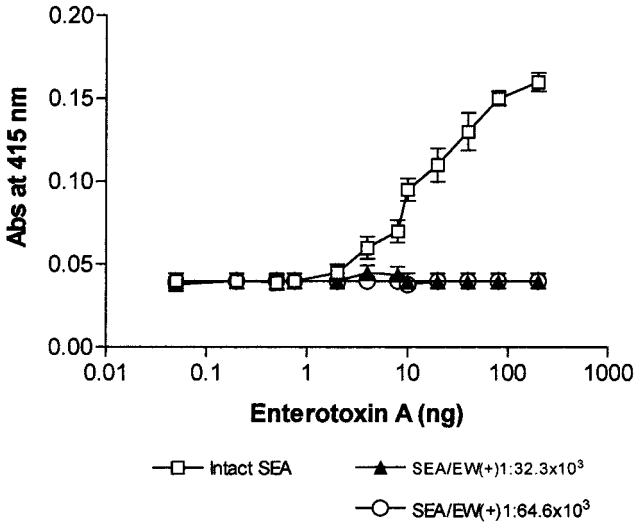

Figure 2. Reduction in the amount of Staphyloccal enterotoxin A after exposure to EW(+) as determined by ELISA. SEA (70 ng, 2.6 pmol) was exposed to 5- or 10-fold volume excess of EW(+) corresponding to 32.3 $\times$ 10^3- or 64.6 $\times$ 10^3-fold molar excess of HOCl in EW(+). Experimental details are given in the text. Data represent mean $\pm$ SD for 5 measurements.

of EW(+) solution, or 19.3 $\times$ 10^3-fold molar excess of HOCl in EW(+) solution, reduced the amount of SEA by 25%. Furthermore, no aggregation of SEA with anti-SEA antibody latex was observed in the RPLA test when SEA was exposed to a 10-fold volume excess of EW(+), or ca. 64.6 $\times$ 10^3-fold molar ratio of HOCl in EW(+). In contrast, SEA was not inactivated by exposure to any volume of EW(−) (Figure 1a).

Addition of SEA (90 μg) to brain−heart infusion culture media clearly caused aggregation in the RPLA test, whereas SEA in culture media exposed to a 100-fold volume excess of EW(+), or ca. 480-fold molar excess of HOCl in EW(+), showed no aggregation. These results indicate that EW(+), when HOCl is present in a sufficient molar excess, can denature the immunoreactive site of SEA even in the presence of organic substances (Figure 1b.). No decrease of SEA was recognized by the exposure of 0.1% NaCl with different molar ratio to SEA (Figure 1c).

Enzyme-Linked Immuno Sorbent Assay (ELISA). Because the RPLA test is a semiquantitative assay with poor sensitivity, we used a quantitative ELISA (Figure 2) to repeat and confirm results obtained from the RPLA test. The detection limit of SEA, measured by absorption at 415 nm, was determined to be 3 ng for samples not exposed to EW(+). However, after SEA was exposed to a 5-fold volume excess of EW(+), or ca. 32.3 $\times$ 10^3-fold molar excess of HOCl in EW(+), no SEA could be detected by ELISA even when the initial amount of SEA was increased to 100 ng.

These results indicate at least that the antigenic determinant of SEA must be denatured upon exposure to EW(+), or that the three-dimensional protein structure of SEA is modified such that the immunoreactivity of SEA with the anti-SEA antibody is lost. Because the anti-SEA antibody used in this study was SEA-specific, the immune reactivity of SEA was definitely lost. Whether the cytotoxicity of SEA is also eliminated remains to be confirmed by cytotoxicity tests in future studies.

Native PAGE. Native polyacrylamide gel electrophoresis of SEA with, or without, exposure to EW(+) or EW(−) is shown in Figure 3. Intact SEA was visualized at around 30 kDa; however, SEA exposure to EW(+) resulted in an indefinite band

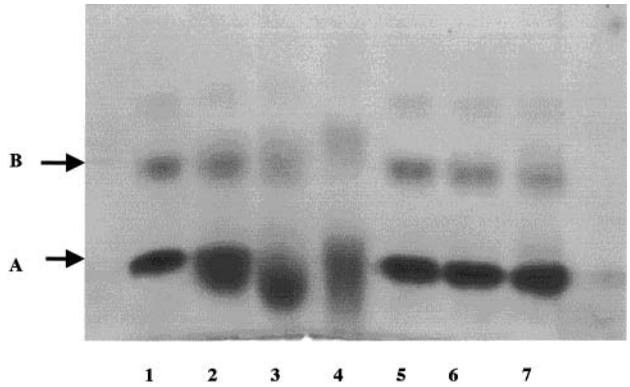

Figure 3. Native PAGE profiles of Staphylococcal enterotoxin A (SEA) after exposure to electrolyzed anodic [EW(+)] or cathodic [EW(−)] solutions. Lane 1, standard SEA; lanes 2–4, SEA exposed to 3-, 5-, and 10-fold volume excess, corresponding to 19.3×10^3-, 32.3×10^3-, and 64.6×10^3-fold molar excess, of HOCl in electrolyzed anodic NaCl solution, respectively; lanes 5–7, SEA exposed to 3-, 5-, and 10-fold volume excess of electrolyzed cathodic solution, respectively. The band indicated by arrow A is SEA and the band indicated by arrow B is BSA. SEA and BSA (ca 20 μg protein, total), with or without exposure to electrolyzed solutions, were applied onto each well for electrophoresis.

Table 1. Change in Amino Acid Composition of Staphylococcal Enterotoxin A after Exposure to an Electrolyzed Anodic NaCl Solution.

	amino acid composition of SEA (mol/M)			
amino acid	reference value[a]	control SEA[b]	EW(+) exposed SEA[c]	percentage loss[d]
Ala	(7)	7.8	6.9	11.5
Arg	(7)	6.6	5.0	24.2
Asx[e]	(36)	37.4	17.3	53.7
Glx[f]	(27)	24.1	15.9	34.0
Gly	(15)	12.9	9.3	27.4
His	(6)	6.4	4.7	25.9
Ile	(10)	8.8	2.7	69.1
Leu	(23)	23.3	17.8	23.6
Lys	(24)	18.6	14.3	23.0
Met	(2)	1.5	0	100
Phe	(8)	7.8	6.3	19.8
Pro	(4)	4.0	3.3	17.2
Ser	(13)	1.0	0.9	11.7
Thr	(16)	5.2	4.4	15.5
Tyr	(16)	13.4	4.1	69.4
Val	(13)	12.6	9.6	24.0
average % loss				34.4

[a] Numerals in parentheses represent the amino acid composition of untreated SEA (mol per 1 M SEA; Huang et al., 1987 (*20*)). [b] Amino acid composition of untreated SEA as determined in our laboratory as mean of 3 measurements. [c] Amino acid composition of SEA after exposure to a 5-fold molar excess of EW(+) for 5 min. [d] Amino acid loss relative to amino acid determination in this laboratory. [e] Asx, aspartic acid + asparagines. [f] Glx, glutamic acid + glutamine.

below and around the 30 kDa region. These data suggest that the EW(+) caused disintegration and fragmentation of SEA (lanes 2 to 4). No changes were observed for the electrophoretic pattern of SEA exposed to EW(−) (lanes 5 to 7). These results clearly indicate that exposure to EW(+) breaks down SEA into peptide fragments.

Amino Acid Analysis. The amino acid composition of SEA with, or without, exposure to EW(+) is shown in Table 1. The amino acid composition of SEA clearly changed after treatment with a 10-fold volume excess of EW(+), or ca. 64.6×10^3-fold molar excess of HOCl in EW(+). It should be noted, however, that the procedure used for amino acid analysis in this study does not give accurate data for tryptophan and cystein, owing to hydrolysis. Except for the content of lysine, serine, and threonine, the amino acid composition of untreated SEA was in good agreement with the reference value reported by Huang et al. (*20*), whereas most amino acids in EW(+)-treated SEA decreased. The reason the content of lysine, serine, and threonine in the SEA (purchased from Sigma-Aldrich, Inc.) used in our experiment differed so much remains unclear. The decreased content of methionine, isoleucine, tyrosine, asparagine, and aspartic acid was particularly marked. Methionine and tyrosine are known to be highly susceptible to oxidation, and methionine is especially sensitive to hypochlorous acid (*29*). But the content of histidine, which is also sensitive to oxidation, did not decrease as much as that of methionine.

From a practical point of view, the results of this study suggest that EW(+), if used properly, can eliminate SEA. Notably, SEA secreted into the extracellular medium from *S. aureus* cells grown in a brain−heart infusion could be also deactivated by EW(+) exposure by the exposure of a large excess of EW(+) with enough HOCl. This is interesting because EW(+) denatures SEA through an oxidative reaction caused by OH radicals and reactive chlorine, and the presence of sulfur-containing amino acids usually inhibits its effect. However, concerning food wholesomeness and soundness, it may not necessarily be appropriate to apply EW(+) to wash food materials. We should be careful to avoid damaging the nutrient values of food materials when applying any treatment, and for the secondary function of food materials as well. As acceptable practical use at present, we propose EW(+) for washing off microbes attached on the surfaces of utensils and piping systems in dairy industries that would endanger food products via contamination with SEA. The effect of EW(+) to disinfect SEA-forming *S. aureus* is sustained for at least several days after electrolysis as long as the product is kept in a cool, dark place under tightly packed conditions (data not shown); however, its effect is lost within several hours when the product was kept in an open container under the sunlight (data not shown). From these results, we strongly propose EW(+) should be used immediately, on-site, after preparation of the product. The presence of transient metals, such as divalent iron, inhibits the effect of EW(+). Detailed information on the adverse effect of transient metals will be reported elsewhere.

If the toxicity of the pathogen is lost or reduced by EW(+), the use of EW(+) might be applicable to the management of food hygiene. It would be interesting to examine whether treatment with EW(+) can impair the ability of *S. aureus* to produce SEA at the genetic level. For example, in cases where the complete disinfection of pathogenic microbes is difficult, the reduction of pathogenicity via a reduction in toxin expression would be useful in preventing outbreaks of food-borne disease. We are currently investigating this subject by PCR.

An interesting finding is that disinfection with hypochlorite (500 ppm available chlorine) is reported to be effective for inactivating the prion that causes Creutzfeldt−Jacob disease (*30*). Although we have not yet confirmed this finding, it would be worth examining whether treatment with electrolyzed anode NaCl solutions is useful for the prevention of bovine spongiform encephalopathy. Electrolyzed anode NaCl solutions might also be effective in disinfecting against hepatitis A virus, as sodium hypochlorite has been shown to reduce the virus by 99.9% (*31*).

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