

## Inactivation of *Escherichia coli* Endotoxin by Soft Hydrothermal Processing<sup>∇</sup>

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**Bacterial endotoxins, also known as lipopolysaccharides, are a fever-producing by-product of gram-negative bacteria commonly known as pyrogens. It is essential to remove endotoxins from parenteral preparations since they have multiple injurious biological activities. Because of their strong heat resistance (e.g., requiring dry-heat sterilization at 250°C for 30 min) and the formation of various supramolecular aggregates, depyrogenation is more difficult than sterilization. We report here that soft hydrothermal processing, which has many advantages in safety and cost efficiency, is sufficient to assure complete depyrogenation by the inactivation of endotoxins. The endotoxin concentration in a sample was measured by using a chromogenic limulus method with an endotoxin-specific limulus reagent. The endotoxin concentration was calculated from a standard curve obtained using a serial dilution of a standard solution. We show that endotoxins were completely inactivated by soft hydrothermal processing at 130°C for 60 min or at 140°C for 30 min in the presence of a high steam saturation ratio or with a flow system. Moreover, it is easy to remove endotoxins from water by soft hydrothermal processing similarly at 130°C for 60 min or at 140°C for 30 min, without any requirement for ultrafiltration, nonselective adsorption with a hydrophobic adsorbent, or an anion exchanger. These findings indicate that soft hydrothermal processing, applied in the presence of a high steam saturation ratio or with a flow system, can inactivate endotoxins and may be useful for the depyrogenation of parenterals, including end products and medical devices that cannot be exposed to the high temperatures of dry heat treatments.**

Endotoxins are lipopolysaccharides (LPS) that are derived from the cell membranes of gram-negative bacteria and are continuously released into the environment. The release of LPS occurs not only upon cell death but also during growth and division. In the pharmaceutical industry, it is essential to remove endotoxins from parenteral preparations since they have multiple injurious biological activities, including pyrogenicity, lethality, Schwartzman reactivity, adjuvant activity, and macrophage activation (2, 9, 12, 13, 25, 32). Endotoxins are very stable molecules that are capable of resisting extreme temperatures and pH values (3, 16, 17, 29, 30, 34, 38). An endotoxin monomer has a molar mass of 10 to 20 kDa and forms supramolecular aggregates in aqueous solutions (22, 39) due to its amphipathic structure, which makes depyrogenation more difficult than sterilization. Endotoxins are not efficiently inactivated with the regular heat sterilization procedures recommended by the Japanese Pharmacopoeia. These procedures are steam heat treatment at 121°C for 20 min or dry-heat treatment for at least 1 h at 180°C. It is well accepted that only dry-heat treatment is efficient in destroying endotoxins (3, 16, 29, 30) and that endotoxins can be inactivated when exposed to a temperature of 250°C for more than 30 min or 180°C for more than 3 h (14, 36). In the production of parenterals, it is necessary to both depyrogenate the final products and carry out sterilization to avoid bacterial contamination.

Several studies have examined dry-heat treatment, which is a very efficient means to degrade endotoxins (6, 20, 21, 26, 41,

42). However, its application is restricted to steel and glass implements that can tolerate high temperatures of >250°C. For sterilization, dry heat treatment tends to be used only with thermostable materials that cannot be sterilized by steam heat treatment (autoclaving). Alternative depyrogenation processes include the application of activated carbon (35), oxidation (15), and acidic or alkaline reagents (27), but steam heat treatment would be an attractive option if it were sufficiently effective. However, the data on the inactivation of endotoxins by steam heat treatment are insufficient and contradictory. It has been reported that endotoxins were not efficiently inactivated by steam heat treatment at 121°C (19, 45). However, Ogawa et al. (31) recently reported that steam heat treatment was efficient in inactivating low concentrations of endotoxin, and that *Escherichia coli* LPS are unstable in aqueous solutions even at relatively low temperatures such as 70°C (see also reference 40). As mentioned above, these reports have shown that although studies have been carried out on the use of steam heat for depyrogenation, there is little agreement on its efficiency.

The U.S. Pharmacopoeia (USP) recommends depyrogenation by dry-heat treatment at temperatures above 220°C for as long as is necessary to achieve a  $\geq 3$ -log reduction in the activity of endotoxin, if the value is  $\geq 1,000$  endotoxin units (EU)/ml (11, 44). Due to the serious risks associated with endotoxins, the U.S. Food and Drug Administration (FDA) has set guidelines for medical devices and parenterals. The protocol to test for endotoxin contamination of medical devices recommends immersion of the device in endotoxin-free water for at least 1 h at room temperature, followed by testing of this extract/eluate for endotoxin. Current FDA limits are such that eluates from medical devices may not exceed 0.5 EU/ml, or 0.06 EU/ml if the device comes into contact with

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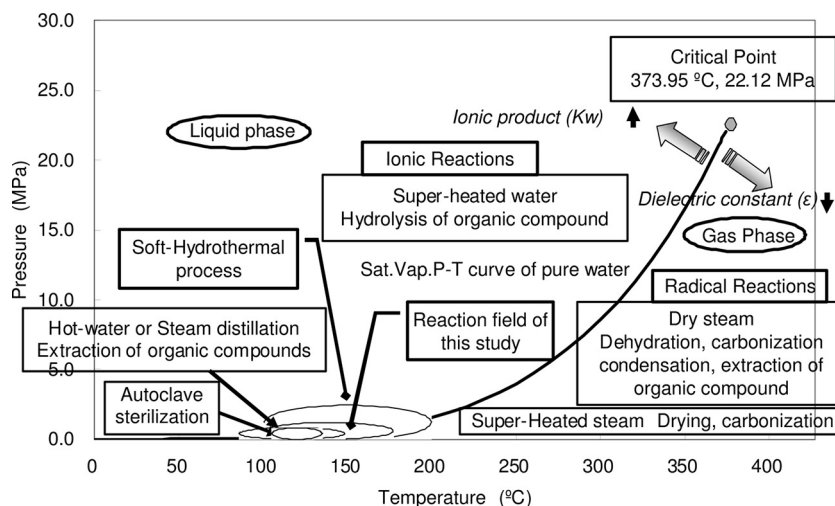


FIG. 1. Reaction field in the pressure-temperature relationship of water. The curve represents the saturated vapor pressure curve. The fields show where the pressure-temperature relationships are conducive to a variety of hydrothermal processing conditions, in which water has a large impact as a reaction medium. Because high-density water has a large dielectric constant and ionic product, it is an effective reaction medium for advancing ionic reactions, whereas water (in the form of steam) on the lower-pressure side of the saturated vapor pressure curve shows a good ability to form materials by covalent bonding. Small changes in the density of water can result in changes in the chemical affinity, which has the potential to advance a range of ionic and radical reactions.

cerebrospinal fluid (43). The term EU describes the biological activity of endotoxins. For example, 100 pg of the standard endotoxin EC-5, 200 pg of EC-2, and 120 pg of endotoxin from *E. coli* O111:B4 all have an activity of 1 EU (17, 23).

Steam heat treatment is comparatively easy to apply and control. If steam heat treatment could reliably inactivate endotoxins, it could be applied with sterilization, reducing labor, time, and expenditure. However, to our knowledge, few studies have addressed steam heat inactivation to determine the chemical and physical reactions that occur during the hydrothermal process, nor have any studies examined the relationship between the steam saturation ratio and the inactivation of endotoxins. Moreover, to date no study has been conducted on steam heat activation of endotoxins with reference to the chemical and physical parameters of the hydrothermal process.

We have developed a groundbreaking method to thermoinactivate endotoxins by means of a soft hydrothermal process, in which the steam saturation ratio can be controlled. The steam saturation ratio is calculated as follows: steam saturation ratio (%) = [steam density ( $\text{kg}/\text{m}^3$ )/saturated steam density ( $\text{kg}/\text{m}^3$ )]  $\times$  100.

The soft hydrothermal process lies in the part of the liquid phase of water with a high steam saturation ratio that is characterized by a higher ionic product ( $k_w$ ) than that of ordinary water. The ionic product is a key parameter in promoting ionic reactions and can be related to hydrolysis. The ionic product of water is  $1.0 \times 10^{-14}$  ( $\text{mol}/\text{liter}$ )<sup>2</sup> at room temperature and increases with increasing temperature and pressure. A high ionic product favors the solubility of highly polar and ionic compounds, creating the possibility of accelerating the hydrolysis reaction process of organic compounds. Thus, water can play the role of both an acidic and an alkaline catalyst in the hydrothermal process (Fig. 1) (1, 37, 46). However, the soft hydrothermal process lies in the high-density water molecular

area of the steam-gas biphasic field (Fig. 1) and is characterized by a lower dielectric constant ( $\epsilon$ ) than that of ordinary water. This process opens the possibility of promoting the affinity of water for nonpolar or low-polarity compounds, such as lipophilic organic compounds (46). We previously reported that most of the predominant aromatic hydrocarbons were removed from softwood bedding that had been treated by soft hydrothermal processing (24, 28).

The purpose of the present study was to evaluate the thermoinactivation of endotoxins by the soft hydrothermal process, by controlling the steam saturation ratio, temperature, and time of treatment. There have been reports that endotoxins were thermoinactivated by steam heat treatment at 121°C in the presence of a nonionic surfactant and at over 135°C in its absence (4, 5, 10), but the minimum temperature for the inactivation of endotoxin remained unknown. This report provides the answer to this question.

## MATERIALS AND METHODS

**Materials.** The USP reference standard endotoxin from *E. coli* O113:H10 was purchased from Seikagaku Biobusiness Co. (Tokyo, Japan) as a powder with a biological activity of 2,000 EU/ml and was used for thermoinactivation experiment and *Limulus* amoebocyte lysate (LAL) assay. Pyrogen-free water was purchased from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). An Endospey ES-24S kit and a standard endotoxin CSE kit, containing the reagent for the LAL assay (8, 18), were purchased from Seikagaku Biobusiness Co.

**Determining the thermoinactivation of endotoxins.** A temperature course of thermoinactivation of endotoxins was measured in a batch process by treating aqueous solutions of endotoxin (challenge concentration of 2,000 EU/ml) with water that was adjusted to the desired steam saturation ratio at the designated temperature. A temperature course of thermoinactivation of endotoxins was also evaluated in a flow system under nonequilibrium conditions by using a flow-type endotoxin inactivation apparatus (Fig. 2) with air-dried endotoxin (also at a challenge concentration of 2,000 EU/ml) treated at the designated temperature. The remaining endotoxin activity was measured by using the LAL assay.

**LAL assay.** The endotoxin concentration in a sample was measured by the chromogenic limulus method (7, 33) with an endotoxin-specific limulus reagent, using Endospey ES-24S and CSE-L as standard endotoxins. The endotoxin-

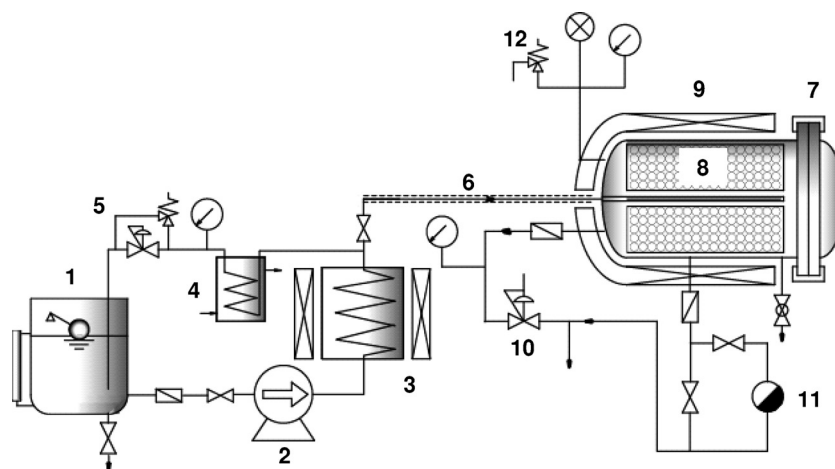


FIG. 2. Schematic diagram showing the flow-type endotoxin inactivation apparatus used for the soft hydrothermal process. Components: 1, water tank; 2, plunger pump; 3, steam generator; 4, condenser; 5, backpressure regulator; 6, line heater; 7, reactor; 8, sample cages; 9, reactor heater; 10, control valve; 11, steam trap; 12, safety valve. The apparatus for the soft hydrothermal process consists of four main components: a cylindrical reactor (400-mm inner diameter, 1,260-mm length, and 158.3-liter volume) equipped with a 9-kW electric heater, a steam generator with a 24-kW electric heater, a backpressure regulator, and a reactor pressure controller. The connecting pipes are constructed of SUS304.

dependent release of *p*-nitroaniline from the chromogenic substrate was recorded by measuring the absorbance at 405 nm. The absorbance at 492 nm was used as a reference to calculate the rate of increase in the difference between the two absorbances per minute. The endotoxin concentration was automatically calculated from a standard curve obtained by a serial dilution of the two standard solutions. Endotoxin concentrations were measured as follows. Each USP reference standard endotoxin was suspended in pyrogen-free water at 2,000 EU/ml (the solubility limit in aqueous solution) by vortexing the sample for 30 min. Then, 10  $\mu$ l of the aqueous solution of endotoxin was added dropwise to a 5-ml vial and subsequently air dried for 24 h in biohazard safety cabinet. The air-dried endotoxin was added to pyrogen-free water, and the vials used in the batch assay were tightly sealed and treated with soft hydrothermal processing. The endotoxin samples were exposed by steam generated inside the vial. A flow-type endotoxin inactivation apparatus (Fig. 2, prototype model; Maeda Seisakusho Co., Ltd, Nagano, Japan) that is able to control the temperature from 100 to 200°C and the pressure from 0.1 to 0.9 MPa was used as an autoclave. After exposure at the designated temperature and time period, the endotoxin samples were cooled, diluted with pyrogen-free water to decrease the concentration of endotoxin to a value in the range of the standard curve, and subjected to the LAL assay using an EG Reader SV-12 (Seikagaku Biobusiness Co.).

**Evaluation of the effect of the steam saturation ratio.** The water (vapor) amount of the designated steam saturation ratio was calculated by using the gas equation of vapor as follows:  $PV = n \times RT$  ( $n = G/M$ ), where  $P$  is the pressure of vapor (in MPa),  $V$  is the volume of the vial (in  $\text{dm}^3$ ),  $n$  is the number of moles,  $G$  is the weight of water (in kg),  $M$  is the molecular weight of  $\text{H}_2\text{O}$  (18.015),  $R$  is the gas constant ( $8.314 \times 10^{-3} \text{ dm}^3 \cdot \text{MPa/K} \cdot \text{mol}$ ), and  $T$  is the temperature of the vapor (in K). The temperature dependency of the thermoinactivation of endotoxins was evaluated at steam saturation ratios of 50, 100, and 1,000% (dry steam, saturated vapor, and superheated water, respectively). The calculated amount of water equivalent to the designated temperature for the 5-ml vials was added as shown in Table 1.

**Statistical procedures.** All results were expressed as the mean  $\pm$  the standard deviation. Parametric comparisons used analysis of variance (ANOVA). The significance of individual differences was evaluated by using the Student *t* test and Tukey's honestly significant differences adjustment if the one-way or two-way ANOVA results were significant. The analyses were performed in a worksheet program (Analytical Tools in Microsoft Excel; Microsoft Office 2003). The statistical significance was set at  $P < 0.05$ .

## RESULTS

**Thermoinactivation of endotoxin by soft-hydrothermal processing with various steam saturation ratios.** To determine the optimal steam saturation ratio, temperature, and reaction time

for the thermoinactivation of endotoxins, we carried out inactivation at a steam saturation ratio of 50, 100, or 1,000% or with the flow system, at 120, 130, or 140°C, for 30 min. The log reduction value (LRV) reflects the thermoinactivation of the endotoxin and was calculated as follows:  $\text{LRV} = \log(\text{challenge concentration of endotoxin}/\text{concentration of endotoxin after processing})$ .

Figure 3 shows that treatment at 120°C for 30 min resulted in LRVs for endotoxins of  $0.51 \pm 0.19$ ,  $1.71 \pm 1.70$ ,  $2.26 \pm 0.15$ , and  $1.60 \pm 0.36$  with a 50, 100, or 1,000% steam saturation ratio or with the flow system, respectively. The LRVs for treatment at 130°C for 30 min with a 50 or 100% steam saturation ratio were  $0.26 \pm 0.08$  or  $1.76 \pm 0.27$ , whereas at 1,000% and with the flow system, the LRVs were  $3.24 \pm 0.13$  or  $3.04 \pm 1.45$ , respectively. According to the USP criteria (44), the latter two conditions achieved depyrogenation ( $\text{LRV} > 3$ ). Similarly, treatment at 140°C for 30 min gave LRVs of  $0.79 \pm 0.14$  and  $2.87 \pm 0.66$  with 50 and 100% steam saturation ratios, respectively, whereas at 1,000% and with the flow system the LRVs were  $4.16 \pm 0.16$  and  $4.41 \pm 1.18$ , respectively. No significant difference in LRVs was observed between 130 and 140°C for 30 min of treatment at steam saturation ratios of 100 or 1,000% or

TABLE 1. Steam saturation ratio and amount of water

Temp (°C)	Calculated amt of water (mg) <sup>a</sup> at a steam saturation ratio of:		
	50% (saturated vapor $\times$ 1/2)	100% (saturated vapor)	1,000% (saturated vapor $\times$ 10)
105	2.0	4.0	40.0
110	2.3	4.6	46.0
120	3.1	6.1	61.0
121	3.2	6.3	63.0

<sup>a</sup> The amount of saturated vapor water was calculated by using a gas equation of vapor equivalent to the designated temperature for a 5-ml vial. The 50% steam saturation ratio was determined by using one-half the amount of water versus the saturated vapor. The 1,000% steam saturation ratio was determined by using 10 times the amount of water versus the saturated vapor.

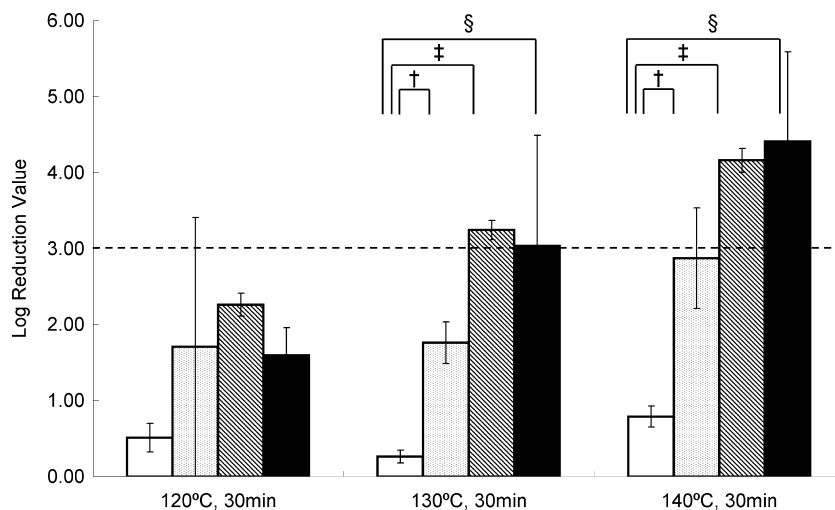


FIG. 3. Temperature course of the inactivation of endotoxin with relation to the steam saturation ratio and to the flow system for 30 min. Inactivation was carried out at 120, 130, or 140°C for 30 min. The data are expressed as means  $\pm$  the standard deviations ( $n = 3$ ). Columns: open, LRV at a 50% steam saturation ratio; stippled, LRV at a 100% steam saturation ratio; cross-hatched, LRV at a 1,000% steam saturation ratio; solid, LRV of the flow system. The broken line represents the USP guideline boundary for depyrogenation with an LRV of  $>3.0$ . Significant individual differences were evaluated by using Tukey's honestly significant differences adjustment if the two-way ANOVA was significant (the symbols †, ‡, and § indicate  $P < 0.05$ ).

with the flow system. In contrast, the differences in LRVs were statistically significant at a steam saturation ratio of 50% ( $P < 0.05$ ). In addition, for 30 min of treatment at 140°C at a 1,000% steam saturation ratio and with the flow system, the endotoxins were completely inactivated ( $LRV > 4$ ). More interestingly, the effect of the steam saturation ratio was statistically significant between 50 and 100%, 1,000%, or the flow system ( $P < 0.05$ ), although it was not significant between 100%, 1,000%, and the flow system. This suggests that even trace amounts of water contribute appreciably to the thermoinactivation of endotoxins.

We then treated the endotoxins at 130°C with a 50, 100, or 1,000% steam saturation ratio or with the flow system for 30, 60, or 90 min. The temperature of 130°C was then selected on

the basis of the results of the first experiment to evaluate the contribution of the steam saturation ratio to the thermoinactivation of endotoxins. After 60 min, endotoxins treated with a 50% steam saturation ratio were not inactivated ( $LRV$  of  $0.50 \pm 0.65$ ), whereas endotoxins treated with a 1,000% steam saturation ratio and with the flow system were inactivated ( $LRV$  of  $3.68 \pm 0.68$  and  $3.77 \pm 0.46$ , respectively) and achieved depyrogenation ( $LRV > 3$ , Fig. 4). Similarly, after 90 min, endotoxins treated with a 50% steam saturation ratio were not inactivated ( $LRV$  of  $1.37 \pm 0.30$ ), although the degree of inactivation was greater than that at 60 min. With treatment at a 100% steam saturation ratio, the endotoxin was almost inactivated ( $LRV$  of  $3.45 \pm 0.75$ ), and treatment at 1,000% and with the flow system completely inactivated the

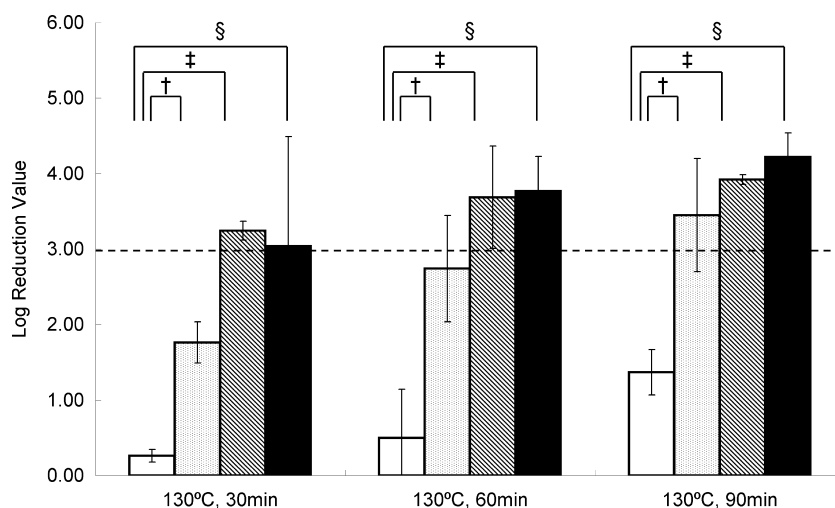


FIG. 4. Time course of the inactivation of endotoxin with relation to the steam saturation ratio and to the flow system at 130°C. Inactivation was carried out for 30, 60, or 90 min at 130°C. The data are expressed as described for Fig. 3.



TABLE 2. Influence of the steam saturation ratio on the inactivation of endotoxin<sup>a</sup>

Temp (°C), time (min)	Mean concn of endotoxin (EU/ml) ± SD at a steam saturation ratio of:			Mean concn of endotoxin (EU/ml) ± SD with the flow system
	50%	100%	1,000%	
120, 30	616.0 ± 321.2	289.5 ± 368.2	10.7 ± 4.6	57.1 ± 38.8
130, 30	1,013.3 ± 164.8	37.6 ± 28.1	1.1 ± 0.2	10.5 ± 14.6
130, 60	677.3 ± 534.9	4.4 ± 4.2	0.6 ± 0.9	0.3 ± 0.4
130, 90	66.7 ± 46.9	1.3 ± 2.0	0.2 ± 0.0	0.1 ± 0.1
140, 30	309.3 ± 100.0	5.6 ± 8.0	0.1 ± 0.0	0.3 ± 0.4

<sup>a</sup> The challenge concentration of endotoxin from *E. coli* O113:H10 was 2,000 EU/ml. Results are the means of three experiments.

endotoxins. In Fig. 4, the same observation can be made as in Fig. 3: the effect of the steam saturation ratio was statistically significant between 50 and 100%, 1,000%, and the flow system ( $P < 0.05$ ), although there was no statistically significant difference between 100%, 1,000%, and the flow system. This suggests that the thermal inactivation of endotoxins was promoted in reaction conditions with a 100% steam saturation ratio or greater and that even a trace increase in water between 50 and 100% steam saturation ratios affected the thermoinactivation threshold. Thus, an LRV of  $>3$  (corresponding to depyrogenation) occurred with treatment at 130°C for 60 min and at 140°C for 30 min for steam saturation ratios of  $>100\%$ . Table 2 lists the endotoxin concentrations observed after treatment with the various soft hydrothermal conditions mentioned above. A steam saturation ratio of 1,000% and the flow system accomplished depyrogenation according to the FDA guidelines (endotoxin concentration of  $<0.5$  EU/ml) (43) at 130°C for 60 min or at 140°C for 30 min. As shown in Fig. 3 and 4, the LRVs of endotoxins treated with the flow system were the same as those of endotoxins treated with a steam saturation ratio of 1,000% for all treatment conditions. With these treatments, especially the batch process at a steam saturation ratio of 1,000%, endotoxin was removed from the aqueous solution in the vial, that is, endotoxin-free water was produced. This suggests that the endotoxins were disaggregated and their molecular structure was degraded by the soft hydrothermal processing, both in the flow system and in the batch system with a high steam saturation ratio. Therefore, soft hydrothermal processing with the flow system contributed appreciably to the thermoinactivation of endotoxins.

## DISCUSSION

Over the past few decades, several studies have examined the thermoinactivation of endotoxins, but no study looked at this mechanism from the standpoint of the steam saturation ratio, that is, the contribution of water. Our aim was to identify the conditions that cause the thermoinactivation of endotoxins. We have shown that thermoinactivation of endotoxins occurred at 130°C for 60 min or 140°C for 30 min when there was a high steam saturation ratio. Gel filtration studies indicated that the endotoxins were separated into three major fractions by steam heat treatment, implying that the endotoxins were partially degraded to a lower molecular size (21). It is reasonable to presume that hydrolysis of the saccharide chain of the

endotoxin occurred. A strong inactivating effect of nonionic surfactants used as an emulsifier or a solubilizer in combination with steam heat treatment was observed by using the LAL assay and the pyrogen test (4, 5). The nonionic surfactants were effective for the inactivation of endotoxin, indicating that the inactivating mechanism might be related to the interaction of surfactant with lipid A. We showed that the threshold conditions for the thermoinactivation of endotoxins was at 130°C for 60 min or at 140°C for 30 min with a steam saturation ratio of at least 100%. Endotoxins were not thermoinactivated under similar conditions (130°C for 60 min or 140°C for 30 min) when the steam saturation ratio was less than 100%. This showed that with a steam saturation ratio greater than 100%, the decomposition of endotoxin molecules, with disaggregation and hydrolysis, contributed to the thermoinactivation of endotoxins, whereas with a steam saturation ratio less than 100% there was no decomposition. Even trace amounts of water between the 50 and 100% steam saturation ratio affected the thermoinactivation of endotoxin. The efficiency of the inactivation of endotoxins increased dramatically as the steam saturation ratio increased in soft hydrothermal processing. It is clear that the participation of water plays an important role in the inactivation of endotoxins by soft hydrothermal processing.

A similar phenomenon was observed in the thermoinactivation of RNase A, a thermostable enzyme. We have clarified that the mechanism of the irreversible thermoinactivation of RNase A is a combination of the hydrolysis of peptide bonds and the deamidation of amino acid residues, which occurs in the presence of a high steam saturation ratio in which the water is characterized by a higher ionic product ( $k_w$ ) than that of ordinary water (data will be presented elsewhere). The results of the present study suggested that thermoinactivation of endotoxins is caused by a combination of the hydrolysis of the saccharide chain and degradation to a lower molecular size by disaggregation of hydrophobic lipid A and that this process occurred at a high steam saturation ratio in which the water has a higher ionic product ( $k_w$ ) and a lower dielectric constant than ordinary water. The ionic product is a key parameter in ionic reactions and can contribute to hydrolysis, whereas the dielectric constant is a key parameter for the binding affinity of lipophilic moieties in endotoxins such as the saturated fatty acids of lipid A. In consequence, the higher ionic product of water results in acidic catalysis, and the lower dielectric constant of water allows it to play the role of a nonionic surfactant (46). The possible mechanism of thermoinactivation of endotoxin in the high steam saturation ratio was that hydrolysis of the ketosidic linkage between Kdo and lipid A part, or glycosidic phosphate or fatty acids, both in lipid A. This study into the feasibility of the thermoinactivation of endotoxins, with the use of the flow-system apparatus for soft hydrothermal processing, may contribute to the depyrogenation of parenteral products, such as tips, tubes, and plasticware. The flow system (with nonequilibrium conditions) had some advantages over the batch system (with equilibrium conditions), since it was more suitable for rapid reactions, and depyrogenation of endotoxins could be accompanied by drying in the flow system (24, 28). We also showed that an aqueous solution containing 2,000 EU of endotoxin/ml was thermoinactivated at 130°C for 60 min or at 140°C for 30 min simply by maintaining the steam saturation ratio above 100% in a batch process. This procedure

should contribute to producing endotoxin-free water readily without the need for current processes, such as LPS affinity resins, two-phase extractions, ultrafiltration, hydrophobic interaction chromatography, ion-exchange chromatography, and membrane adsorbers. In this process, the reaction mediator was only water, which is entirely nontoxic. Harmful organic compounds were not required and were not generated as by-products. The results indicate that soft hydrothermal processing in the presence of a high steam saturation ratio or with a flow system has a strong ability to inactivate endotoxins and is one of the most advanced techniques for the depyrogenation of parenterals, including end products and medical devices that cannot be exposed to the high temperatures used for dry-heat treatment.

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