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Heat and High-Pressure Treatments on *In Vitro* Digestibility and Allergenicity of Beef Extract

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Abstract The digestibility of heat- or high-pressure-treated beef extracts was evaluated with an *in vitro* simulated gastric digestion model and each sample also underwent immune assay to detect its antigenicity with the sera of beef allergic patients. Heat treatment of the beef extracts considerably decreased their digestibility, whereas high-pressure treatment at 200 MPa improved their digestibility compared with the control, but the difference was not significant. The digestibility of the high-pressure-treated beef extract was generally higher than that of the heat-treated samples. Depending on the degree of digestion, the degree of antigenicity of the main beef allergens decreased. On the basis of these results, we hypothesized that the allergenicity of beef could be eliminated if the allergenic proteins are sufficiently digested in the digestive organ, leading to the suggestion that the digestibility of allergenic proteins must be improved in food processing. In conclusion, high-pressure processing is a more acceptable food processing technique for beef considering its digestibility.

Keywords: digestibility, allergenicity, heat, high-pressure, beef extract

Introduction

In our previous report (1), we found that both bovine serum albumin (BSA) and bovine gamma globulin (BGG) played an important role in the allergenicity of beef. We also reported the cross-reactivity between beef- and cow's milk-allergens. Since BSA has also been regarded to be a milk allergen (2, 3), its antigenic properties have been studied by many researchers (4-7). Peters *et al.* (4) have suggested that there is significant variation in the antigenicity of each different peptide fragment of BSA prepared through limited proteolytic treatment. Atassi *et al.* (6) and Habeeb and Atassi (7) have reported that BSA has repeating identical antigenic reactive sites. Wahn *et al.* (5) have suggested the presence of at least four antigenic IgE-binding sites on BSA.

Various food processing techniques have been applied to foods in order to eliminate their allergenic proteins or to reduce their levels. The effects of heat treatment on food allergenic proteins have been widely studied by many researchers. Heat treatment reduced the sensitization of the beef, even if the treatment was less effective on pure BSA under domestic conditions (8-10), whereas in other reports heat treatment showed negative results. For example, the allergenicity of shrimp allergens (11) and cow's milk allergens (12) increased with heat treatment. In another interesting report, the allergenicity of the egg white was not eliminated by heat treatment alone, whereas the allergenic protein (ovomucoid) was wholly depleted by rinsing the egg white in saline solution and distilled water after heat treatment (13). Enzymatic treatment has also been used for eliminating or reducing food allergenic proteins. Watanabe *et al.* (14) succeeded in the development of hypoallergenic rice by enzymatic treatment of the grains. Tsumura *et al.* (15) have reported the study of hypoallergenic soybean protein using enzymatic hydrolysis.

The absorption of undigested protein and/or polypeptides in the adult digestion organs is very rare but is a considerable problem in the immature gut of the newborn, which explains why food allergies are prevalent in children (16). Thus, it is important to improve the digestibility of allergenic proteins to reduce their allergenicity. The effect of digestion on the allergenicity of foods by gastric enzymes *in vitro* (17) and the effect of physical treatments on digestibility of food (7, 18) have been studied. Homogenizing, blending, and freeze-drying processes partially improve the digestibility of lamb meat (18, 19). Both proteolytic digestion and physical treatment (heat, homogenization, and freeze-drying) reduce the potential allergenicity of beef (20). Cooking is a critical step to obtain a hygienically safe product. On the other hand, cooking can affect the proteolytic activity of gastrointestinal enzymes as a result of protein structural changes due to heat denaturation (21, 22). The high-pressure treatment has recently come to be considered a useful food processing technique and its efficiency in the treatment of meat has been reported by several research groups, including Suzuki *et al.* (23-25). However, to date studies on the effects of high-pressure treatment on food digestibility and allergenicity have not been undertaken.

This study therefore investigated the effect of heat and high-pressure treatments on the digestibility of beef extract using *in vitro* digestion experiment, and each digested sample also underwent immune assay with the sera of the beef allergic patients in order to analyze its antigenicity.

Materials and Methods

Sera from beef-allergic patients In our previous report (1), we suggested two types of beef allergic patients: type 1 patients who react to both BSA and BGG, and type 2 patients who react to only BGG. In this study, the two kinds of sera of six beef allergic patients were used. These

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sera were kindly provided by Dr. Masatomo Matsuno of Yoshida hospital (Niigata, Japan) for this study.

Preparation of the beef extract After removing the fat and connective tissue, 10 g of the shoulder part of a Holstein beef carcass (one day after slaughter and stored at -20°C) was finely cut by scissors and homogenized three times using a Nissei homogenizer (AM 11; Nihon Seiki Co., Tokyo, Japan) for 5 sec with 100 mL of a 20 mM sodium phosphate buffer (pH 7.4). The homogenate was centrifuged at 20,000×g for 15 min. The supernatant was filtered through Whatman No. 2 filter paper. All procedures were conducted at 4°C. The protein concentration of the extract was determined by the biuret method standardized with BSA (26) and adjusted to 5 mg/mL.

Heat and high-pressure treatment of beef extract The beef extract in a cap-tube underwent heat treatment (60 and 100°C) for 10 min. High-pressure treatment of the beef extract followed the procedure of Homma *et al.* (27). The sample sealed in a polyethylene bag was pressurized at 200, 400, and 600 MPa at 5-7°C for 5 min using an isostatic processor (NBIP; Nikkiso Isostatic Processor, Tokyo, Japan).

***In vitro* digestion of the beef extract** Digestion in the gastric model was carried out according to the method of Astwood *et al.* (17) with a slight modification. Beef extract was digested with pepsin in the ratio of 1:90 (enzyme to sample; w/w) at pH 2.5. After the peptic attack, a solution containing 0.5 mg/mL of trypsin was subsequently added to the sample in the ratio 1:36 (w/w) at pH 7.3. Proteolysis was carried on at 37°C in a water bath shaken at 100 beats/min. The reaction was stopped by trichloroacetic acid (TCA) (final 10%) in order to determine the total amount of free peptide. The reaction was also stopped by pepstatin A (for peptic attack) and leupeptin (for tryptic attack) to undergo sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme-linked immunosorbent assay (ELISA).

TCA-soluble products determination TCA-soluble products of each sample were determined by measuring its absorbance at 280 nm with a spectrophotometer (UV mini 1240; Shimadzu, Kyoto, Japan).

SDS-PAGE SDS-PAGE was carried out according to the method of Laemmli (28) with slight modification, using 4% stacking gel and 10% separating gel. Samples were each suspended in an SDS-sample dilutor (0.25 M Tris-HCl buffer, pH 6.8, containing 10% SDS, 4% 2-mercaptoethanol, 20% glycerol, 0.05% bromo-phenol blue, and 10 mM EDTA). Each sample was denatured by heating at 100°C for 2 min. The gel was stained with a solution containing 0.025% Coomassie brilliant blue (CBB) R-250, 50% methanol and 5% acetic acid, and then destained with 7.5% acetic acid and 5% methanol.

ELISA ELISA was performed according to the method of Engvall and Perlmann (29) with slight modification. Each prepared beef extract was diluted in a 50 mM sodium carbonate buffer (pH 9.6; 1:500 v/v), and 50 mL of each diluted extract (0.01 μg/μL) per well was coated on a 96-well ELISA plate (9018; Costar, NY, USA). The plate was incubated overnight at 4°C, washed with phosphate-buffered saline (PBS; 20 mM sodium phosphate, 150 mM NaCl, 2.7 mM KCl, pH 7.4), blocked for 1 hr with a 2% gelatin (Bio-Rad Lab., Hercules, CA, USA) PBS solution, and then washed three times with PBST (PBS + 0.05% Tween-20). The patient's serum diluted in 1% gelatin-PBS (1:5 v/v) was added to the plate (50 μL per well). The plate was incubated for 2 hr at 25°C, and washed four times with PBST. Subsequently, the 2nd antibody (anti-human IgE peroxidase conjugate; Sigma, St. Louis, MO, USA.) was added to the plate. The plate was washed five times with PBST, and developed with an ABTS peroxidase substrate system (KPI, Gaithersburg, MD, USA) before being read with a plate reader (M680; Bio-Rad Lab.) at 405 nm.

Immunoblotting Immunoblot analyses were carried out according to the method of Towbin (30) with slight modifications. After SDS-PAGE, the proteins were transferred from the gel onto a PVDF membrane (162-0176; Bio-Rad Lab.) with a transfer buffer consisting of 25 mM Tris, 192 mM glycine and 5% methanol. The PVDF membrane was blocked for 2 hr at 25°C in a 2% gelatin-PBST solution. The blocked membrane was rinsed with PBS and then incubated overnight in PBST containing the patient's serum (diluted 1:5 v/v) at 25°C. The membrane was then washed three times with PBST. Subsequently, the membrane was incubated in PBST containing a 1:500 dilution of alkaline phosphatase (AP) conjugated goat anti-human IgE (Tago Immunologicals, Camarillo, CA, USA) as 2nd-antibody for 90 min at 25°C. After washing three times with PBST and once with PBS, AP was detected with an AP conjugate substrate kit (170-5056; Bio-Rad Lab.). In order to check the efficiency of the electrotransfer, the proteins on the PVDF membranes were stained with 0.1% CBB R-25 and then 99% methanol, and destained with 50% methanol and 5% acetic acid.

Results and Discussion

Effects of heat treatment on digestibility and allergenicity To closely mimic the human gastric digestion

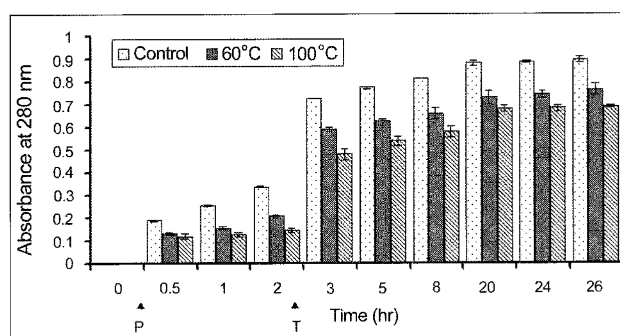

Fig. 1. TCA-soluble products during *in vitro* digestion of heat-treated beef extracts. P, start of peptic digestion; T, start of tryptic digestion. Heat treatments of beef extracts were conducted in a cab-tube for 10 min.

system, mammalian gastric (pepsin) and pancreatic (trypsin) enzymes were used. Figure 1 shows the digestibility of the heat-treated beef extract, expressed as the amount of free peptide liberated during the enzymatic attack. The control beef extract was rapidly hydrolyzed, and then the total free peptide (TCA-soluble products) increased more rapidly than that of the heat-treated samples in all stages of enzymatic attack. The digestibility was gradually decreased with increasing heating temperature. A similar result has been reported by Restani *et al.* (18, 19), indicating that enzymatic digestion of the meat samples is strongly restricted by heat treatment (steam cooking). These results were also supported by their SDS-PAGE patterns (Fig. 2). As shown in the SDS-PAGE patterns of the 60°C sample (Fig. 2B), BGG and, to an even greater extent, BSA were less digested than other proteins. In fact, the BSA band remained even after 24 hr digestion for the heated samples (Fig. 2B and 2C). These results indicate that the major beef allergen BSA was more stable against enzymatic attack than other proteins in the heat-treated beef extract. Astwood *et al.* (17) have suggested that major food allergens were stable to digestion in the gastric model (using simulated gastric fluid).

Each digested sample underwent immune assay with the sera of beef allergic patients in order to evaluate its allergenicity. Figure 3A shows the ELISA results of the non-treated control and heat-treated samples for a type 1 allergic patient during the digestion. The sample heated at 100°C could not be subjected to ELISA due to its aggregation (evident in its immunoblotting result of Fig. 4). Even after enzymatic attacks of 8 or 24 hr, the heat-treated samples maintained their allergenicity of 37 and 23%, respectively, whereas the control sample lost most of its allergenicity after 8-hr enzymatic attack (Fig. 3B). These ELISA results corresponded well with the immunoblotting results (Fig. 4), which indicated that the samples at 60 and 100°C had a specific reaction even after 24 hr (Fig. 4B and 4C), whereas the control sample did not (Fig. 4A). Similar results were obtained in the experiment with type 2 beef allergic patients (data not shown). The ELISA patterns indicated that the allergenicity considerably decreased after 2-hr digestion. The beef allergenicity was largely retained for the 2-hr digestion, presumably because of the presence

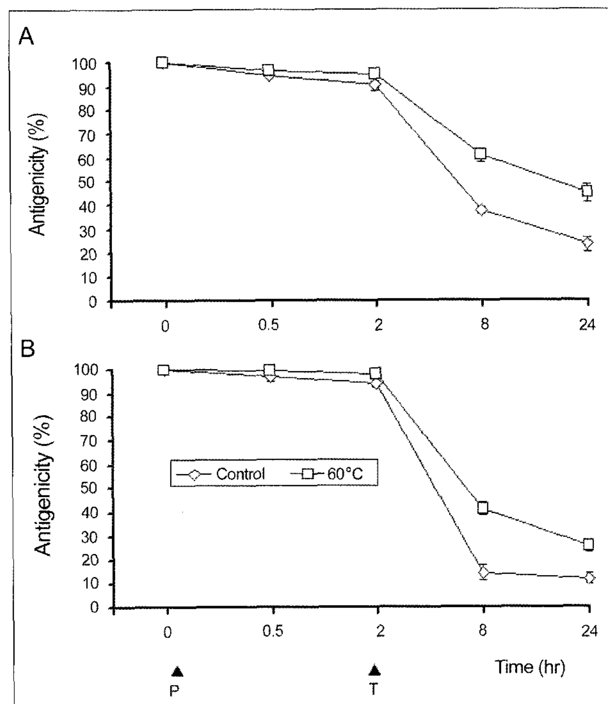

Fig. 3. ELISA of heat-treated beef extracts for the serum of a beef allergic patient during *in vitro* digestion. A, Result with the serum of a type 1 patient; B, result with the serum of a type 2 patient. P, start of peptic digestion; T, start of tryptic digestion. Antigenicity was established by calculating the percentage of the binding activity of the control sample.

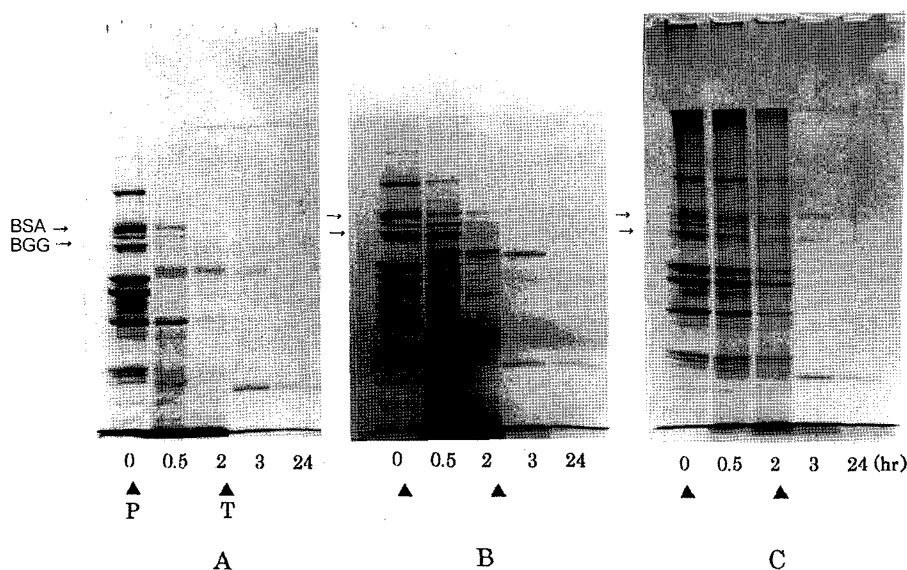

Fig. 2. SDS-PAGE (10%) results of heat-treated beef extracts during *in vitro* digestion. A, Untreated control sample; B, heat-treated sample at 60°C for 10 min; and C, heat-treated sample at 100°C for 10 min. P, start of peptic digestion; T, start of tryptic digestion.

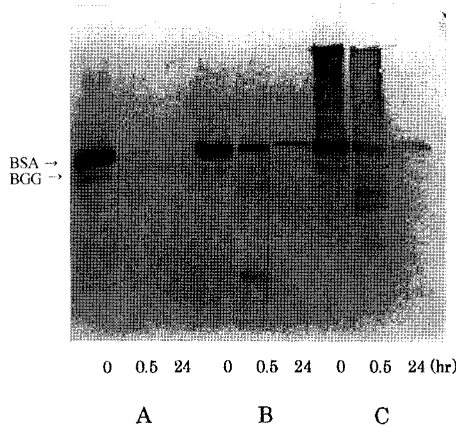

Fig. 4. Immunoblot analyses (10% gel) of heat-treated beef extracts for the serum of a beef allergic patient during *in vitro* digestion. A, Untreated control sample; B, heat-treated sample at 60°C for 10 min; C, heat-treated sample at 100°C for 10 min.

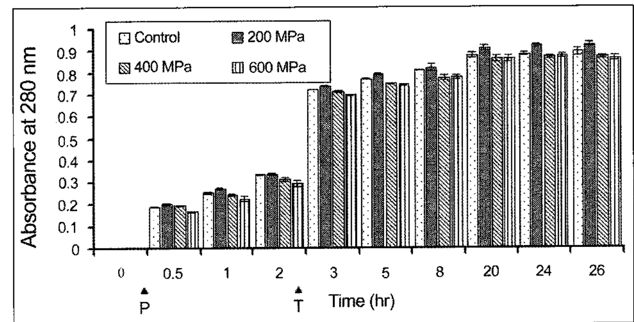

Fig. 5. TCA-soluble products during *in vitro* digestion of high-pressure-treated beef extracts. P, start of peptic digestion; T, start of tryptic digestion. High-pressure treatments were conducted in a polyethylene bag at 5-7°C for 5 min.

of several antigenic IgE-binding sites on BSA and BGG. In fact, there are several reports of BSA showing repeating identical antigenic reactive sites (6, 7) and another report of at least four antigenic IgE-binding sites on it (5). Further studies are needed to confirm this assumption.

Effects of high-pressure treatment on digestibility and allergenicity Figure 5 shows the digestibility of the high-pressure-treated beef extract, expressed as the amount of TCA-soluble products of each sample during digestion. The sample pressurized at 200 MPa showed a slightly increased digestibility compared with the control sample during either pepsin or trypsin treatment. The digestibility of the 400 and 600 MPa samples was lower than that of the control sample. Figure 6 shows the SDS-PAGE patterns of the pressurized samples. Main beef allergenic molecules (BSA and BGG) in 200 and 400 MPa samples were mostly digested for 2 hr (Fig. 6A and 6B). When we compared the effects on digestibility of the heated and pressurized beef extracts, the digestibility of the pressurized samples (Fig. 5 and 6) was higher than that of the heated samples (Fig. 1 and 2) during the entire digestion experiment. This phenomenon was more evident in the BGG molecules when the results of each SDS-PAGE trial of heated and pressurized samples were compared (Fig. 2 and 6). Even if the high-pressure level for the treatment of beef extract increased to 600 MPa, the digestibility of the allergenic proteins was higher than that of the 60 and 100°C samples during the same period of the experiment, indicating that the digestibility of high-pressure-treated beef extract was generally higher than that of the heat-treated sample. These results indicated that the high-pressure treatment of meats might be a useful food processing technique considering their digestibility.

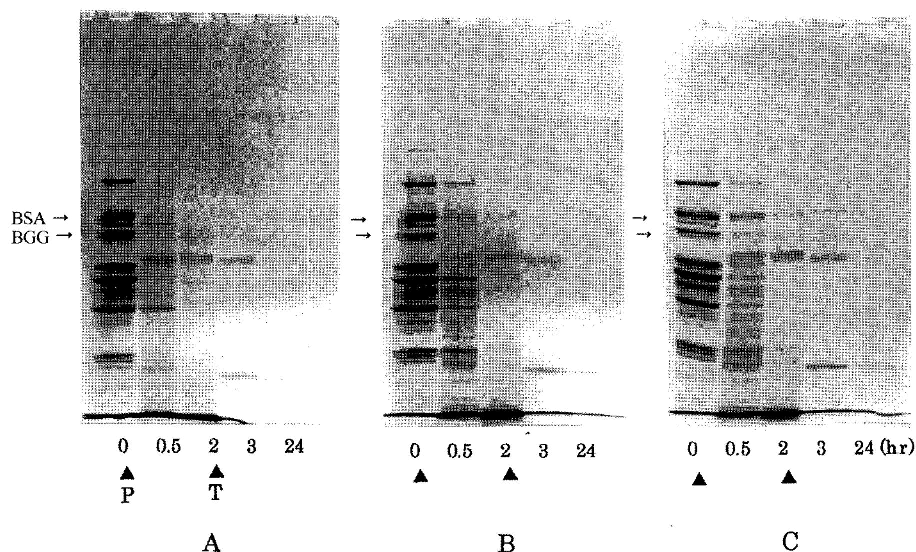

Fig. 6. SDS-PAGE (10%) of high-pressure-treated beef extracts during *in vitro* digestion. A, High-pressure-treated sample at 200 MPa; B, high-pressure-treated sample at 400 MPa; and C, high-pressure-treated sample at 600 MPa. P, start of peptic digestion; T, start of tryptic digestion. High-pressure treatments were conducted in a polyethylene bag at 5-7°C for 5 min.

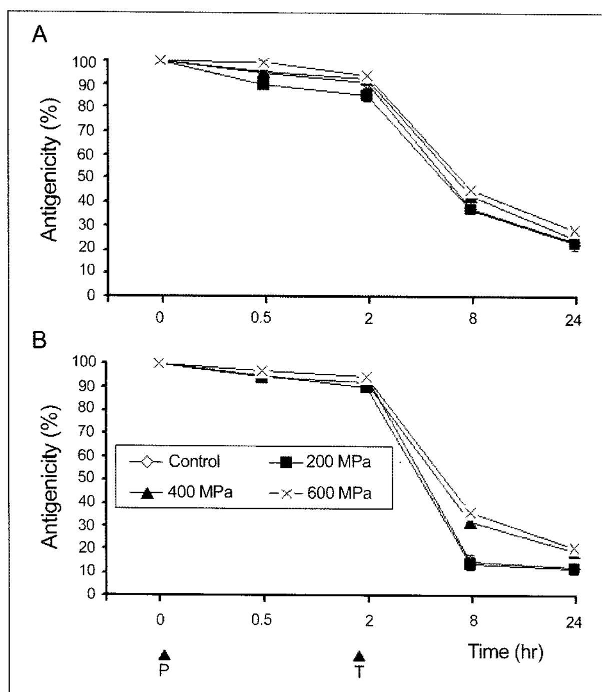

Fig. 7. ELISA of high-pressure-treated beef extracts for the serum of a beef allergic patient during *in vitro* digestion. A, Result with the serum of a type 1 patient; and B, result with the serum of a type 2 patient. P, start of peptic digestion; T, start of tryptic digestion. Antigenicity was established by calculating the percentage of the binding activity of the control sample.

Figure 7A shows the ELISA results of beef extract treated with high-pressure for a type 1 allergic patient during digestion. The allergenicity of the control and 200 MPa samples mostly disappeared after 8-hr digestion, as shown in Fig. 7. After 8 and 24 hr of digestion, the 400 MPa sample maintained its allergenicity of 32 and 19% and the 600 MPa sample maintained its allergenicity of 35 and 20%, respectively. However, these scores were lower than those of the heat-treated samples at either 60 or 100 °C (Fig. 4). Similar results were obtained in the experiment with type 2 beef allergic patients, as shown in Fig. 7B.

Heat treatment for the beef extract decreased its digestibility in the simulated gastric digestion model, whereas high-pressure treatment at 200 MPa improved its digestibility, but not significantly. These differences between heat and high-pressure treatment on digestibility could be explained by the resistance of the heat-denaturized protein to attack by the endopeptidases (pepsin and trypsin) used in this experiment due to its coagulated structure. On the contrary, the unfolding of the protein structure induced by high-pressure treatment increased the surface area available to enzymatic contact, thereby helping the enzyme to attack the protein.

On the basis of these results, we suggest that sufficient digestion of the allergenic proteins in the gastric digestion system will eliminate the allergenicity, which suggests the importance of improving the digestibility of allergenic proteins in food processing. The results also support the use of high-pressure treatment in combination with other food processing techniques as being more useful than heat or high-pressure treatment alone for improving the digestibility of beef allergenic proteins. Further research into applications of such combined treatments is necessary.

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