We often eat heat-coagulated (H-C) food proteins, but there have been few studies on the allergenic activity of H-C proteins after digestion and absorption in vivo. To show that H-C protein is not an allergen after digestion, mice were used to investigate the digestion and absorption of the protein through the intestinal epithelium into portal blood employing immunoblotting and competitive inhibition ELISA. Ovalbumin (OVA) was used as the model protein, and H-C OVA was prepared by heating a 5% OVA solution for 15 min in boiling water. Antigenic OVA was not detected in the soluble fraction of gastrointestinal contents or the portal blood of mice administered H-C OVA. Also, voluntary physical activities, as an assessment of anaphylaxis, were monitored for 15 h using OVA sensitized mice. Compared to the voluntary physical activities of sensitized mice without any load, no decrease in activity was observed in the group administered H-C OVA, but a significant decrease in activity was found in the mice administered unheated OVA. These results strongly indicate that H-C OVA does not retain allergenic properties.

**Key words:** heat-coagulated protein; digestion; absorption; allergenic activity; voluntary physical activity

Dietary antigens crossing the gastrointestinal barrier to the blood in experimental animals are well documented. In 1955 Gruskay and Cooke reported that normal children absorbed dietary antigen (OVA), and there was increased uptake during and after acute gastroenteritis. In allergic children and in celiac disease, ovalbumin has also been shown to be taken up in increased amounts. β-lactoglobulin and OVA, which are derived from cow’s milk and eggs, frequently appeared in the breast milk when ingested by lactating women. These food antigens found in breast milk include intact proteins and sometimes cause allergic reactions in exclusively breast-fed infants. Husby et al. found 10.5 μg OVA/ml in the sera from seven of eight healthy adults 2–3 h after ingestion of 60 ml raw egg and 500 ml milk. These reports suggest that the intestinal barrier was permeable to macromolecules.

Some proteolytic enzymes have been used to digest raw globular proteins to evaluate allergenic activity. Stability against pepsin digestion is a significant and valid parameter that distinguishes food allergens from non-allergens, but a clear relationship has not been established between digestibility measured in vitro and protein allergenicity. The validity of digestion stability as a criterion for assessing whether protein is allergenic has encountered some criticism in recent years. In these reports, the allergenic properties of many raw food proteins were investigated as to human intake and proteolytic hydrolysis.

Dietary proteins are usually prepared in heat-denatured and coagulated forms. Consequently, it is necessary to confirm the allergenic activity of the heat-coagulated proteins after digestion in vivo. As indirect proof that heat-denatured proteins are not allergenic, Peng et al. reported that heat-denaturation of egg white proteins abrogated the induction of oral tolerance of specific Th2 immune responses in mice. On the other hand, Urisu et al. reported that 37 of 38 subjects (97%) who immediately manifested positive reactions to freeze-dried egg white did not respond to heated egg white in double-blind, placebo-controlled tests.

It is unclear how heat-coagulated proteins digested in the gastrointestinal tract and hydrolyzed peptides, with or without allergenic activity, are absorbed into portal blood. Hence, this study investigated the digestion of OVA as a model protein and its absorption through the intestinal epithelium into portal blood. Subsequently, the allergenic activity of the absorbed products was assessed utilizing sensitized mice.

**Materials and Methods**

**Preparation of heat-coagulated OVA.** OVA was freshly prepared from the white of White Leghorn eggs by the ammonium sulfate precipitation method. A 5% solution of OVA in PBS was heated in boiling water for 15 min, and a homogenizer (Model Polytron PT 10-35, Kinematica, Tokyo, Japan) was used to prepare a H-C
OVA solution orally administered to the mice. Disappearance of the allergenic activity of soluble OVA was confirmed by immunoblotting using rabbit anti-OVA serum (data not shown).  

**Digestion of unheated and H-C OVA in vivo.** Female BALB/c mice weighing about 25 g (n = 24) were used in this experiment. They were maintained in a 20 ± 2°C air-conditioned room that was lighted from 8 AM to 10 PM. A standard diet and water were provided ad libitum. After a 24-h fast, a 5% solution of unheated (n = 12) or H-C OVA (n = 12) in 0.5 ml PBS was orally administered with a syringe under light anesthesia. Fifteen and 30 min later (n = 6/group), celiotomies were quickly performed under anesthesia and portal blood samples were collected. Serum was obtained by centrifugation at 3,000 rpm for 15 min at 4°C and diluted twofold with glycerol. The stomachs and small intestines were removed, and the small intestine was divided into six equal parts. The stomach contents and each small intestine segment were extracted with 1 ml of PBS. The stomach extracts were separated into soluble and insoluble fractions by centrifugation at 3,000 rpm for 15 min. All samples were stored at −30°C or lower. The experimental procedures employed followed the guidelines set forth in the *Care and Use of Animals in the Field of Physiological Sciences* approved by the Council of the Physiological Society of Japan.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting. SDS–PAGE was carried out with a 15% or 17.5% gel. After sample migration, bands were stained with 0.1% (w/v) Coomassie brilliant blue R-250 according to the method of Laemmli. The proteins separated on the gel were transferred electrophoretically onto a nitrocellulose sheet (0.45 μm, Advantec Toyo, Tokyo, Japan) by the method of Towbin et al. The sheet was blocked overnight with 3% BSA/TBS (bovine serum albumin/tris-buffered saline, pH 7.5), and then incubated for 1 h in a 1% BSA/TBS solution containing rabbit anti-OVA serum with mild shaking at RT. After the sheet was washed three times with TBS-T, a peroxidase conjugated goat anti-rabbit IgG in 1% BSA/TBS solution was added and incubated for 1 h with mild shaking at RT. The sheet was then washed with TBS-T three times and TBS two times. The protein bands with reactivity to the specific antisera were activity-stained for peroxidase using 4-chloro-napththol for 20–30 min.

**Competitive binding inhibition ELISA.** OVA concentration in the portal blood was determined by ELISA competitive inhibition using a standard curve derived from OVA (50 μg/ml) dissolved in blood serum of control mice. Flat-bottomed micro-titer plates (Nunc, Denmark) were pre-coated with 100 μl of OVA solution (10 μg/ml) and incubated overnight at 4°C. Immediately before use, the wells were washed with phosphate buffer saline containing 0.05% Tween 20 (PBS-T). A 1% BSA/PBS-T solution was added to each well and incubated for 30 min at 37°C. Each 15 μl of serum and standard solution was serially diluted with PBS after agitation and added to the same amount of anti-OVA in 1% BSA/PBS-T. After incubation overnight at 4°C, the mixture was added to washed plates and incubated for 3 h at 37°C. After each well was washed three times with PBS-T, 100 μl of peroxidase conjugated goat anti-rabbit IgG in 1% BSA/PBS-T solution was added to each well and incubated for 3 h at 37°C. Each well was washed three times with PBS-T and 100 μl of o-phenylenediamine in citrate-phosphate buffer (0.4 mg/ml) containing 0.03% H2O2 was added. The reaction was stopped with 50 μl of 5 N H2SO4 solution after 20 min at room temperature. Color development was measured photometrically at 492 nm.

**Sensitization of mice against OVA.** Female BALB/c mice weighing 25 g (n = 12) were used in this experiment. The mice were intraperitoneally injected with 10 μg OVA and 1 mg aluminum hydroxide in 200 μl phosphate-buffered saline six times at an interval of 10 d. Blood was obtained weekly from a vein under light ether anesthesia.

**Measurement of anti-OVA specific IgE.** Specific anti-OVA IgE in mouse serum was evaluated by enzyme linked immuno-sorbent assay. A hundred μl of OVA solution (10 μg/ml) was added to each well of flat-bottomed micro-titer plates and incubated overnight at 4°C. The wells were then washed with phosphate buffer saline containing 0.05% Tween 20 (PBS-T). A 1% BSA (bovine serum albumin)/PBS-T solution was added to each well and incubated for 30 min at 37°C. Then 100 μl of mouse serum was diluted 50 times with 1% BSA/PBS-T solution, applied to a well, and incubated for 3 h at 37°C. After each well was washed three times with PBS-T, 100 μl of peroxidase conjugated goat anti-mouse IgE in 1% BSA/PBS-T solution (10−3) was added to each well and incubated for 3 h at 37°C. Each well was treated as described above.

**Forced movement load.** Before the experiment, all sensitized BALB/c mice were exercised during a 3-d acclimation period. Then the mice orally administered proteins were forced to run on a treadmill set to 15 m/min and 10% gradient for 30 min.

**Measurement of voluntary physical activity.** Allergen orally administered BALB/c mice (OVA 13.5 mg/mouse) with or without forced treadmill running were housed in cages with a running wheel, and the number of revolutions per h was measured for 15 h from 8 PM to 11 AM using a counting and accumulating printer (Natsume Seisakusho, Tokyo, Japan).

**Statistical analysis.** The total revolutions for 15 h and
hourly revolutions were expressed as the mean of the six mice in each group. Statistical analysis of the data comparing H-C OVA and unheated OVA groups was performed using the unpaired Student t-test. P values for significance were set at 0.05 or 0.01.

**Results and Discussion**

*Digestion in the stomach and small intestine*

To examine whether the digestion of H-C OVA regains antigenic activity in the process of digestion in the gastrointestinal tract, H-C OVA was orally administered to the mice. The SDS–PAGE and immunoblotting profiles of the soluble and insoluble fractions of stomach contents from mice administered H-C OVA and unheated OVA are shown in Fig. 1. OVA and many peptide bands reactive to anti-OVA serum were found in the insoluble fraction of the stomach contents of H-C OVA mice. Similar results were obtained in the insoluble fraction of unheated OVA groups. This finding suggests that both heated and unheated protein were coagulated in the low pH environment of the stomach. This phenomenon was more clearly observed at 15 min than at 30 min after administration. The soluble fractions in stomachs of mice administered heated OVA exhibited OVA and hydrolyzed peptides clearly immunoblotted by anti-OVA serum, but no such immunosensitive bands were observed in the H-C OVA group. Immuno-reactive peptides remained insoluble in the stomach and no immuno-detectable peptides were in the soluble fraction of mice given H-C OVA. The difference in time interval, 15 or 30 min after H-C OVA administration, was not a factor affecting the disappearance of antigenic OVA in the soluble fractions of the stomach.
Each of the six luminal contents for all the mice were extracted with 1 ml of PBS. Equal volumes of the soluble contents from corresponding segments of the six mice in each group were combined and mean SDS–PAGE and immunoblotting patterns were obtained, as shown in Fig. 2. Several bands immunoblotted with anti-OVA serum were observed in the soluble fraction of mice administered unheated OVA. Fifteen min after unheated OVA was administered, the fourth segment yielded OVA and low molecular weight bands that strongly immunostained. At 30 min, strongly stained bands were observed in the second, fifth, and sixth segments. In contrast, no immuno-reactive bands were detected in any segment of mice administered H-C OVA. These results clearly indicate that antigenic OVA was not present in the small intestine of mice administered H-C OVA. On the other hand, 30 min after unheated OVA was administered, immunostained OVA and peptides were found in the lower small intestine.

Absorption of antigenic OVA into portal blood

On the basis of the above finding, that OVA was not detected in the soluble fractions of the gastrointestinal contents of mice administered H-C OVA, the presence of OVA in portal blood was examined to confirm that heat-coagulated OVA was not absorbed into the body. As shown in Fig. 3, no immunoblotted bands were observed in the portal blood of mice administered H-C OVA. On the other hand, an immunostained band of OVA was observed in portal blood 15 min after the administration of unheated OVA. Similar patterns were also observed in two portal blood samples 30 min after administration.

OVA concentrations in portal blood were determined by ELISA competitive inhibition (typical profiles are shown in Fig. 4). In those mice administered unheated OVA, a large difference was found in detectable OVA in those blood samples. No antigenic OVA was found in five and four mice 15 and 30 min respectively after unheated OVA administration. A few micrograms (1–2.5 μg/ml) per ml of antigenic OVA were calibrated in one mouse’s serum after 15 min and in two mice after 30 min. These sera corresponded to the immunoblotting patterns, as shown in mouse no. 3 after 15 min and mice nos. 1 and 2 after 30 min Fig. 3.

As shown in the in vivo experiments described above, no OVA antigenic activity was detected in the soluble fraction of the gastrointestinal tract or portal blood of mice given H-C OVA. To clarify further the decrease in allergenic activity of H-C OVA, anaphylaxis of sensitized mice was measured using voluntary physical activity as the criterion. Voluntary physical activity was measured by recording the number of revolutions run. We reported previously that physical activity markedly decreased in lysozyme-sensitized mice after they were given an allergen orally.18)

Assessment of H-C OVA allergenic activity using wheel-running as a measure of anaphylaxis

After anti-OVA specific IgE values were significantly increased by administering repeated doses of OVA (Fig. 5), the sensitized mice were separated into two groups administered either unheated OVA or H-C OVA. Then running activities were measured for 15 h during a normal activity period (Fig. 6). Sensitized mice were monitored for 15 h during a normal activity period.
Sensitized mice without any load application ran approximately 10,000 revolutions/15 h, and a similar number was obtained for those administered H-C OVA. The mean for those administered unheated OVA decreased to about 7,000, but the difference was not significant. Then the mice were forced to exercise on a treadmill set to 15 m/min and 10% gradient for 30 min after they were administered unheated or H-C OVA. Following compulsory treadmill running, voluntary wheel running was monitored hourly for 15 h. A significant difference in wheel-running activity was observed between the unheated and H-C OVA mice (p < 0.01).

The number of revolutions run was monitored hourly from 8 PM to 11 AM, as shown in Fig. 7. The mice administered unheated OVA ran significantly less for the first 7 h than those administered H-C OVA. Allergen administration combined with compulsory exercise
reduced the physical activity of the sensitized mice, and this effect continued for about 7 h. A decrease in physical activity in sensitized mice is an indicator of the presence of allergen, as described previously. 19)

Our result, that no antigenic activity of OVA was detected in the portal blood of mice administered H-C OVA and that anaphylaxis symptoms were repressed in sensitized mice administered H-C OVA, indicate that heat-coagulated protein is not an allergen. Astwood et al. reported that important food allergens were not digested in simulated gastric fluid, and that this stability is a significant and valid parameter for distinguishing food allergens from non-allergens. Prospective testing for the allergenicity of proteins is valuable, and the next step that assessing the allergenic activity of proteins causing anaphylaxis that is necessary. We have reported that the physical activity of lysozyme-sensitized mice after oral allergen challenge clearly decreased as compared with the activity of unsensitized mice. The assessment system for anaphylaxis using the voluntary physical activity of sensitized mice described here is valuable for estimating the allergenic activity of the denatured-coagulated protein before an oral challenge test to sensitized humans to confirm the allergenicity of a protein.

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References


