

High-temperature (121 °C) treatment of soy protein isolate with water partially decomposes constituent amino acid residues, which reduces the nutritional value of protein as an amino acid source and alters social novelty recognition in mice

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Abstract

High-temperature (HT) treatments above 100°C are important in food processing; however, their effects on the nutritional value of moist heat-treated proteins are less understood. Therefore, this study examined the impact of HT at 121°C on soy protein isolate (SPI) in water. After 20 min of HT treatment, most amino acid levels in SPI decreased significantly, despite minimal nitrogen loss. HT also increased insoluble protein and larger soluble peptides after *in vitro* digestion. Mice administered HT-treated SPI showed lower serum levels of free amino acids, thereby reducing bioavailability. Long-term feeding with 20% HT-treated SPI impaired social novelty recognition and decreased peripheral serotonin levels, while anxiety-like behavior, object exploration, sociability, and serum tryptophan remained unaffected. These findings indicate that HT treatment not only reduces the nutritional value of SPI but may also generate Maillard-type compounds or modified peptides that interfere with tryptophan metabolism and social behavior.

INTRODUCTION

High-temperature (HT) treatment above 100°C is widely used in food processing. Foods packed in airtight containers, such as cans, bottles, retort pouches, and retort trays, are heated at temperatures of 121°C or higher for an appropriate period, typically using a retort or autoclave, allowing them to be stored at room temperature. Microbial cell membranes, enzymes, and spores in foods are denatured using HT treatment, a process also known as sterilization¹. The production of retort-packaged products is on the rise², including infant foods, possibly due to no need to add synthetic preservatives. Additionally, it has been reported that protease inhibitors in foods such as soy trypsin inhibitors are inactivated by HT treatment³. Thus, HT treatment improves the digestibility of food protein, enhancing the nutritional value⁴. In contrast, some studies have reported that HT treatment of protein in dry form modifies amino acid residue and decreases nutritional value^{5–7}. HT treatment of food proteins generates dehydroalanine residues from serine and cysteine residues, which react with lysine and cysteine residues to form lysinoalanine and lanthionine crosslinks, respectively⁸. This reaction decreases available lysine and cysteine and the digestibility of proteins. HT treatment of powdered foods, such as skim milk and soy protein isolate (SPI), in an autoclave at 121°C has been demonstrated to decrease protein efficiency ratio and protein digestibility-corrected amino acid score in an animal model^{9,10}. However, in practice, HT processing of foods is often performed on prepared moist foods, rather than on powders¹¹. There is only limited information on the effects of HT treatment of protein in wet form on nutritional values and metabolic fate. In a preliminary experiment, SPI was suspended in water to achieve a 10% concentration and then heated at 121°C for 20 min in an autoclave, simulating common food processing conditions. Rats fed the 10% HT-treated SPI diet for two weeks exhibited significantly reduced feed efficiency compared with those fed non-treated SPI (Supplementary Tables S1 and S2), along with modest changes in serum biochemical parameters (Supplementary Table S3). These findings prompted further investigation into the nutritional and behavioral consequences of HT-treated SPI in a mouse model. In this study, the effects of the HT treatment of SPI in the presence of water on amino

acid composition, *in vitro* digestibility, and *in vivo* amino acid bioavailability were examined. Furthermore, the effects of long-term feeding of a 20% HT-treated SPI diet on the growth and behavior of mice were examined.

RESULTS

Chemical Changes in HT-treated SPI

Figure 1A shows the SDS-PAGE patterns of non-treated and HT-treated SPI. The HT-treated sample exhibited unclear protein bands and uneven staining across the gel, particularly at the bottom of the lane, suggesting that HT treatment resulted in nonspecific cleavage, polymerization, or chemical modification. Kjeldahl analysis revealed that HT treatment slightly reduced the crude protein content of SPI to 98.3% (Fig. 1B). Furthermore, amino acid analysis revealed a substantial reduction in total protein content to 90.3% by HT treatment (Fig. 1C). Amino acid analysis revealed significantly lower SPI protein content after HT treatment than nitrogen-based estimates ($p < 0.001$). The content of amino acids, except for Met and Trp, in HCl and NaOH hydrolysates of HT-treated SPI, respectively, was significantly lower than that of non-treated SPI (Fig. 1D).

To address the reason for the reduction of amino acid residues in SPI by HT treatment, effects of HT treatment on reducing sugars and ammonia levels were examined, as reducing sugars can react with the amino group by the Maillard reaction and ammonia can be liberated from the amino and amide groups. The contents of glucose and galactose derived from oligosaccharides such as raffinose and stachyose contaminating SPI were significantly reduced by HT treatment (Fig. 2A). No significant changes occurred in the levels of sugar components of glycoproteins, such as mannose, glucosamine, and fucose. The total reduction contents of monosaccharide in SPI by HT treatment were approximately 10 $\mu\text{mol/g}$ (Fig. 2B), which was far less than the reduction of amino acids (approximately 1 mmol/g).

To investigate the contribution of deamination and deamidation reactions to the reduction of amino acid residues, the free ammonia content was quantified. Free ammonia content increased to 20 $\mu\text{mol/g}$ of SPI by HT treatment (Fig. 3A), which was less than one-fiftieth of the reduction in amino acid residues observed in the hydrolyzed SPI (Fig. 3B). HCl hydrolysis of the non-treated SPI generated approximately 1.80 mmol of free ammonia/g of SPI. This ammonia can be derived from amide in the side chains of Asn and Gln residues in proteins. HT treatment significantly increased ammonia concentrations in the HCl hydrolysate of SPI to 1.98 mmol/g (Fig. 3B). Thus, HT treatment increased ammonia in the HCl hydrolysate by over 120 $\mu\text{mol/g}$, corresponding to approximately 12% of the loss of amino acid residues due to HT treatment.

Nondigestible Proteins and Peptides in HT-treated SPI

The following analysis examined how HT treatment influenced the digestibility of SPI and promoted the formation of digestion-resistant proteins and peptides. HT-treated and non-treated SPIs were digested

with pepsin and pancreatin. The pepsin-pancreatin digest of non-treated SPI was completely soluble, whereas the digest of HT-treated SPI contained 53.0 ± 9.9 mg of insoluble material/g of SPI ($n = 3$). Amino acid analysis revealed that insoluble material contained protein (Supplementary Figure S1). This indicates that HT treatment generates insoluble proteins that are resistant to endoproteinases. To assess the resistance to exopeptidases, the soluble fractions of the pepsin-pancreatin digest were further digested with carboxypeptidase A and leucine aminopeptidase. The peptides in the endoproteinases-exopeptidases digests of HT-treated and non-treated SPI were analyzed using SEC. Elution of the peptide was monitored using amino acid analysis of the HCl hydrolysate of the SEC fraction (Fig. 4). HT treatment of SPI increased the ratio of peptides with molecular weights ranging from 200 to 500 Da in the digest, while decreasing peptides with molecular weights less than 200 Da. These results indicate that HT treatment modifies the amino acid residues of proteins in SPI, resulting in the formation of digestion-resistant peptides that remained even after exopeptidase treatment.

Mice Serum Amino Acids Levels After Single Administration of SPIs

The effects of HT-treated SPI administration on temporal changes in serum free amino acid concentrations were subsequently investigated. As shown in Fig. 5, serum levels of Thr, Ala, and Met were significantly lower 60 min after administration of HT-treated SPI than those of the non-treated SPI. Trp levels were significantly lower at 120 minutes after administration of HT-treated SPI. In contrast, no significant differences were observed in serum amino acid concentrations between mice administered with HT-treated SPI and those with non-treated SPI at 30 min after administration.

Effects of HT-treated SPI Feeding on Behavior

Behavioral outcomes were evaluated after a six-week feeding period using the experimental diets described in Table 1, in which 20% of the total protein source was replaced with HT-treated SPI or non-treated SPI. Particular attention was given to anxiety-related activity, recognition memory, and social interaction.

Table 1
Diet composition.

Components (g)	Non-treated SPI	HT-treated SPI
Cornstarch	39.7	39.7
Non-treated SPI	20.0	-
HT-treated SPI	-	20.0
Alfa-corn starch	13.2	13.2
Sucrose	10.0	10.0
Soybean oil	7.0	7.0
Cellulose	5.0	5.0
Mineral mixture	3.5	3.5
Vitamin mixture	1.0	1.0
Choline tartrate	0.25	0.25
L-cystine	0.3	0.3
<i>t</i> -Butylhydroquinone	0.0014	0.0014
Total	100	100

No significant differences in body weight were observed between mice fed non-treated SPI and those fed HT-treated SPI at weeks 1, 3, 5, and 7 (data not shown).

Anxiety-like behavior was assessed using the open-field test and the elevated plus maze test. In both tests, no significant differences were observed between the two groups in terms of time spent in the center zone or on the open arms, the number of entries, or total locomotor activity (Fig. 6). In the object recognition test, no significant differences were found between groups in time spent sniffing the objects or in locomotor activity during the acquisition session (Fig. 7). In the recognition session, mice from both groups spent significantly more time sniffing the novel object than the familiar one. The recognition index did not differ significantly between the two groups. To evaluate social behavior, the three-chamber social test was performed (Fig. 8). In session 1 (sociability phase), both groups spent more time in the chamber containing a stranger mouse (Stranger 1) than in the empty chamber, and spent more time sniffing Stranger 1 in the cage than in the empty cage. No significant differences were observed between the groups in any parameters during session 1. In session 2 (social novelty phase), mice fed non-treated SPI spent significantly more time in the chamber containing a novel mouse (Stranger 2) than in the chamber with the familiar mouse (Stranger 1) and spent significantly more time sniffing Stranger 2. In contrast, mice fed HT-treated SPI showed no significant differences in time spent in the two chambers or in sniffing duration between Stranger 1 and Stranger 2.

Effects of HT-SPI Feeding on Serum Serotonin and Trp Levels

Serum tryptophan and serotonin levels were assessed after the same six-week feeding period with a 20% HT-treated SPI diet to determine whether dietary modification affected peripheral serotonin metabolism and social behavior.

After seven weeks of feeding experimental diets, serum Trp levels showed no significant difference between mice fed HT-treated SPI and those fed non-treated SPI. In contrast, serum serotonin levels were significantly lower in HT-treated SPI-fed mice than in non-treated SPI-fed mice (Fig. 9).

DISCUSSION

High-temperature (HT) treatment above 120°C is widely used in food processing, particularly for sterilizing moist foods such as canned, bottled, and retort-pouched products^{1,2}. The chemical and nutritional consequences of such moist HT treatment remain less well characterized despite extensive reports on dry heating effects^{6,7}. In this study, we used a 10% aqueous suspension of SPI heated at 121°C for 20 min to model moist heat sterilization and to evaluate its effects on amino-acid composition, digestibility, and biological consequences.

SPI contains approximately 90% protein and 4–6% carbohydrates and minerals per dry matter. Under the present conditions, HT treatment resulted in a ~ 10% reduction in amino acid residues, to a level similar to that observed with dry heating, while nitrogen loss was limited to only 1.3% (Fig. 1B and C). This discrepancy suggests that most amino acid residues were chemically modified rather than volatilized. Dry heating has been reported to liberate ammonia from proteins¹². To minimize ammonia loss before analysis, SPI samples were sealed during heating and immediately frozen. HCl was added to the frozen samples to stabilize volatile compounds. Nonetheless, a small amount of nitrogen was lost, suggesting the formation and loss of volatile nitrogen compounds such as pyrazines, which are poorly ionized even in acid¹³. However, the sum of nitrogen loss and ammonia production by HT treatment accounted for only approximately 15% of the loss of the total amino acid residue (Figs. 1 and 3), suggesting that most lost residues may have been converted into non-volatile nitrogen compounds. Hydrolysis of proteins with HCl releases ammonia from the acid amides of Asn and Gln residues. HT treatment liberated an additional 0.2 mmol/g of ammonia during HCl hydrolysis of SPI, equivalent to approximately 20% of the loss of amino acid residues. This suggests that HT treatment induces the formation of amides or imines, such as Schiff bases, which can release ammonia through acid hydrolysis. Since reducing sugars in SPI were decreased by HT treatment (Fig. 2), carbonyl compounds generated by the Maillard reaction may have reacted with amino groups to produce imines.

The present study revealed that most of the amino acid residues were significantly reduced by HT treatment of SPI. Heat and alkali treatments of proteins are known to induce the formation of lysinoalanine or lanthionine from specific residues such as lysine, cysteine, and serine, which decreases the availability of essential amino acids. SDS-PAGE analysis of HT-treated SPI showed reduced staining

intensity and a smeared lane (Fig. 1A), a pattern consistent with extensive chemical modification, aggregation, or partial peptide bond cleavage, rather than a single specific reaction. Taken together with the broad loss across many amino acid residues (Fig. 1D), these observations are most consistent with partial hydrolysis of protein that liberates amino groups; some liberated amino groups likely underwent secondary reactions to form amides, imine type adducts (e.g., Schiff bases), or crosslinks such as lysinoalanine and lanthionine. Such nonspecific hydrolysis, combined with secondary modifications, can explain reductions in residues beyond those with reactive side-chain amino groups (for example, Lys). Additionally, other chemical modifications occurring during HT treatment, including oxidation and decarboxylation, may contribute to the generation of additional non-standard or digestion-resistant products.

HT treatment generated insoluble protein in SPI after *in vitro* digestion with pepsin and pancreatin. Additionally, HT treatment increased the proportion of exopeptidase-indigestible peptides in the soluble fraction of the pepsin-pancreatin digest (Fig. 4). These observations indicate that HT treatment promotes the formation of both insoluble and soluble digestion-resistant products. Amino-acid analysis showed that these resistant products still contain normal amino acids, since HCl hydrolysis released expected amino-acid profiles from both insoluble and soluble fractions (Supplementary Figure S1 and Fig. 4). Consequently, the amount of non-bioavailable amino acids in HT-treated SPI likely exceeds the portion directly destroyed by HT treatment ($\approx 10\%$). Consistent with impaired immediate bioavailability, total serum free amino-acid levels 60 min after oral administration of HT-treated SPI were $\sim 25\%$ lower than after non-treated SPI (Fig. 5). In preliminary feeding trials, mice given a 10% protein-restricted diet containing HT-treated SPI for two weeks showed reduced feed efficiency compared with mice fed the non-treated SPI diet (Supplementary Table S2), consistent with loss of available amino acids after HT treatment. Similar reductions in protein digestibility have been reported for other heating conditions, including studies on dry-heated SPI in broilers^{14–16} and human diets rich in Maillard reaction products¹⁷.

It has been reported that N-terminal glutamine residues in peptides can undergo intramolecular cyclization between the amino group and the side-chain amide to form a pyrrolidone structure (pyroglutamyl residue; pyroGlu) during heat processing¹⁸. Oligopeptides containing pyroGlu residues are resistant to exopeptidase digestion¹⁹. In addition to pyroglutamyl peptides, peptides bearing other HT-induced modifications—such as deamidation, deamination, decarboxylation, or crosslink formation—may also become resistant to proteolytic digestion. HT treatment has been reported to alter protein allergenicity by changing epitope structures, with possible increases or decreases in allergenic potential^{20,21}. Although allergenicity was not examined in the present study, the generation of chemically modified, digestion-resistant peptides raises the possibility of altered allergenicity and warrants further immunological evaluation.

In a seven-week feeding study using a 20% protein diet, mice fed HT-treated SPI did not show reduced feed efficiency compared with those fed non-treated SPI, likely because total dietary protein remained

sufficient to support normal growth. Under these conditions, however, mice given HT-treated SPI exhibited a selective impairment in social novelty recognition (Fig. 8), whereas object recognition and anxiety-like behaviors were unaffected (Figs. 6, 7). After an 18-h fast at the end of the experiment, serum serotonin concentrations measured in peripheral (abdominal venous) blood were reduced by approximately 50% in the HT-SPI group relative to controls (Fig. 9). Peripheral serotonin-deficient rats generated by tryptophan hydroxylase 1 knockout show altered anxiety-like behavior and changes in brain gene expression despite preserved central serotonin²², suggesting that reduced peripheral serotonin in HT-SPI-fed mice may contribute to the observed behavioral alterations, particularly in social novelty recognition. Peripheral serotonin is synthesized from tryptophan by enterochromaffin (EC) cells in the gut; however, serum tryptophan did not differ between groups after fasting (Fig. 9). Therefore, impaired serotonin synthesis in EC cells, rather than diminished tryptophan availability, presents a plausible explanation. Yano et al. have reported that EC serotonin synthesis can be modulated by gut microbial metabolites²³; accordingly, modified amino acids or digestion-resistant peptides generated by HT treatment of SPI, or their microbial metabolites, are plausible mediators that could affect EC serotonin synthesis and related amino-acid metabolic pathways.

In conclusion, this study demonstrated that HT treatment (121°C, 20 min) of SPI reduced the recoverable total amino-acid content after HCl hydrolysis by approximately 10%. HT treatment also significantly increased free ammonia and acid-labile ammonia released by HCl hydrolysis, consistent with deamination, deamidation, and imine formation via Maillard reaction. These modifications promoted the formation of poorly digestible peptides and insoluble protein products, further reducing the nutritional value of SPI. Moreover, long-term administration of HT-treated SPI to mice decreased peripheral serotonin levels and was associated with impaired social cognitive behavior. Because moist HT treatment is widely used in the food industry for canned, bottled, and retort-pouched products—including infant formula—further research is needed to (i) elucidate the chemical structures of modified amino acids and peptides formed by HT treatment, (ii) determine their effects on digestion, microbiota, and enteroendocrine function, and (iii) assess the impact of consuming HT-treated foods on human nutrition, development, and behavior.

MATERIALS AND METHODS

Preparation of HT-treated SPI

SPI (Fujipro new-E, contains approximately 90% protein and 4–6% carbohydrates and minerals per dry matter) was a kind gift from Fuji Oil (Osaka, Japan). SPI was mixed with distilled water to a final concentration of 10% (w/w). The paste (10 mL, pH 7.12) was sealed in a 40 mL vial (CV-400, Pierce, Thermo Fisher Scientific, Waltham, MA, USA) and heated at 121°C for 20 min in an autoclave (SS-320; Tomy Seiko, Japan). The products were then freeze-dried and powdered. The powder was used as an HT-treated SPI.

Chemicals.

Mineral mixture (AIN-93-MX) and vitamin mixture (AIN-93-VX) were purchased from MP Biomedicals (Solon, OH, USA). Amino acids standard mixture (Type H), acetonitrile (high-performance liquid chromatography (HPLC)-grade), triethylamine, phenyl isothiocyanate, ammonium acetate, trifluoroacetic acid (TFA), choline tartrate, and *t*-butylhydroquinone were purchased from Fuji Film Wako Pure Chemical (Osaka, Japan). Soybean oil, cellulose, alfa-corn starch, sucrose, L-cystine, pancreatin, and thymol were purchased from Nacalai Tesque (Kyoto, Japan). Porcine pepsin, leucine aminopeptidase, and carboxypeptidase A were purchased from Merck (Darmstadt, Germany). 1-Phenyl-3-methyl-5-pyrazolone (PMP) was obtained from Dojindo (Kumamoto, Japan). All other reagents were of analytical grade or better.

In Vitro Protease Digestion

One gram of SPI and HT-treated SPI were suspended in 150 mL of 0.1 M HCl. Ten milligrams of pepsin were added and incubated at 37°C for 3 h. The reaction mixture was mixed with 10 mL of 1 M Tris-HCl buffer, pH 8.0, and the pH was adjusted to 8.0 by adding drops of 1 M NaOH. Forty milligrams of pancreatin were added and incubated at 37°C for 24 h. The pepsin-pancreatin digest was clarified through centrifugation at $5,000 \times g$ for 15 min. The residue was washed twice with water and then freeze-dried. The supernatant was diluted to 500 mL with water and used as a pepsin-pancreatin soluble sample. Leucine aminopeptidase (5 μ L) and carboxypeptidase A (5 μ L) were added to aliquots of the supernatant (500 μ L, adjusted at pH 7.5) and incubated at 37°C for 24 h. Enzymes in the digest were removed by passing the sample through an Amicon Ultra 0.5 mL 3k (Merck) filter using centrifugation at $12,000 \times g$ for 20 min.

Size Exclusion Chromatography

Aliquots of peptidase digest were clarified by passing a Cosmonice filter (0.45 μ m, Nacalai Tesque). Two hundred microliters of the filtrates were subjected to size exclusion chromatography (SEC) using a Superdex Peptide 10/300 GL (GE Healthcare, Chicago, IL, USA). Elution was performed using 0.1% TFA containing 30% acetonitrile at a flow rate of 0.5 mL/min. Fractions were collected every 1 min. Fractionation was repeated three times, yielding 1.5 mL of each fraction.

Amino Acid Analysis

HT-treated SPI (10.0 mg, dry weight) and insoluble residues obtained after pepsin–pancreatin digestion were hydrolyzed under sealed high-temperature conditions. Samples were placed in 40-mL Pierce vials (CV-400; Thermo Fisher Scientific) fitted with Mininert® valves. Then, 1.0 mL of 6 M HCl was added, the vial atmosphere was evacuated using a vacuum pump, the valve was closed, and the vials were hydrolyzed at 150°C for 1 h. After cooling, hydrolysates of bulk samples were diluted to 10.0 mL with Milli-Q water, filtered (0.45 μ m), and processed for amino-acid derivatization and HPLC analysis according to Bidlingmeyer et al.²⁴ with slight modifications²⁵.

Aliquots of SEC fractions were processed using the same sealed-vial hydrolysis method. The samples were first dried in glass tubes (50 mm $\times$ 4 mm i.d.), then transferred into 40-mL Pierce vials containing

1.0 mL of 6 M HCl. The vial atmosphere was evacuated using a vacuum pump, the valve was closed, and the samples in the inner tubes were hydrolyzed by exposure to HCl vapor at 150°C for 1 h. The resulting hydrolysates were then reconstituted and analyzed as described above.

Tryptophan in protein was measured after alkaline hydrolysis; SPI and HT-treated SPI were hydrolyzed under alkaline conditions, and Trp content was quantified using reversed-phase HPLC at Japan Food Research Laboratories (Tokyo, Japan). Free amino acids in SEC fractions and mouse serum were determined after protein removal: aliquots were mixed with three volumes of ethanol, centrifuged at $10,000 \times g$ for 5 min, and the supernatants were collected for analysis. The total nitrogen content of proteins was measured using the Kjeldahl method.

Reducing Sugar Analysis

Sugars in the sample were hydrolyzed with 2.5 M TFA. The sample (10 mg) was placed into the 40-mL vial with 2 mL of 2.5 M TFA and hydrolyzed under vacuum at 100°C for 6 h. The hydrolysate was made up to 5 mL with water and used as a sample. Standard solutions of monosaccharide (100 mM in water) were freshly prepared and further diluted with 75% ethanol to the appropriate concentrations and derivatized with PMP using the method of Honda et al.²⁶. PMP was dissolved in methanol (0.5 M) and mixed with the same volume of 0.3 M NaOH. Samples and standard solutions (10 μ L) were dried in a tube (1.5 mL) and then mixed with 60 μ L of a PMP – alkaline mixture and heated at 70°C for 30 min. The reaction was terminated by adding 200 μ L of 0.1 M HCl and 200 μ L of 100 mM ammonium acetate buffer, pH 6.0. To remove the non-reacted PMP, liquid – liquid partitioning was performed. Chloroform (200 μ L) was added, the mixture was vortexed vigorously, and then centrifuged at $10,000 \times g$ for 5 min. The aqueous layer was collected and filtered using a Cosmonice Filter before HPLC analysis. The derivatives in 10 μ L of filtrate were separated using a Superspher 100 RP-18 (e) (4 μ m, Merck) at 45°C using a binary gradient. Solvent A consisted of 150 mM ammonium acetate buffer, pH 6.0, containing 5 % (v/v) acetonitrile; solvent B was 60% (v/v) acetonitrile. The gradient profile was as follows: 0–5 min, B 25%; 5–20 min, B 25–30%; 20–30 min, B 100%; 30–43 min, B 25%. The flow rate was 0.6 mL/min. Elution was monitored by measuring absorbance at 254 nm.

Sodium Dodecyl Sulfate–polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

SPI samples (10 mg) were mixed with 1 mL of Laemmli sample buffer containing β -mercaptoethanol and heated at 95°C for 5 min. Proteins were separated on 12% polyacrylamide gels under reducing conditions using standard SDS-PAGE protocols²⁷. Electrophoresis was performed at 120 V for 60 min, followed by staining with Coomassie Brilliant Blue R-250.

Ammonia Quantification

SPI (100 mg) was suspended in 1 mL of distilled water and autoclaved in a sealed polypropylene microtube at 121°C for 20 min. To prevent ammonia evaporation, the sealed sample was frozen, and then 100 μ L of 1 M HCl was added to the frozen sample. After melting the frozen sample, ammonia in

the sample was converted to ammonium chloride. Aliquots (100 μ L) of this solution were diluted with 900 μ L of distilled water and centrifuged at $10,000 \times g$ for 5 min. The supernatant was further clarified through ultrafiltration using an Ultrafree-MC with a molecular weight cutoff of 5,000 (Merck Darmstadt, Germany) under centrifugation at $10,000 \times g$ for 20 min. The filtrates were analyzed using ion chromatography (DX-300, Dionex, Sunnyvale, CA) equipped with an IonPac CS14 column and a cation self-regenerating suppressor (CSRS-I). All were obtained from Dionex. The eluent was 10 mM methanesulfonic acid. The flow rate was 0.8 mL/min, and the detection was performed using electrical conductivity. A self-regenerating solution (water) was delivered to the suppressor under pressure at 15 psi.

Preparation of Diets

SPI was also suspended in water and freeze-dried in the same manner as HT-treated SPI without autoclaving and used as non-treated SPI. Experimental diets were prepared according to the recommendations by the National Institute of Nutrition in 1993 (AIN-93G). As shown in Table 1, HT-treated SPI or non-treated SPI was mixed with other ingredients to achieve a 20% by weight replacement of milk casein in the original AIN-93G.

Animal Experiments

All animal experiments were conducted in accordance with institutional guidelines and were approved by the Animal Care Committee of Nara Medical University (approval nos. 12850, 12881, 13089, 13090).

Male C57BL/6 J mice aged 3 weeks were purchased from Japan SLC (Hamamatsu, Japan). Four mice per cage were housed under standard laboratory conditions (23°C, 55% humidity, and a 12-hour light-dark cycle). Mice had access to a standard laboratory chow (CE-2, CLEA Japan, Tokyo) and water ad libitum for one week. After the acclimatization period, the mice were fed an experimental diet containing either non-treated SPI ($n = 12$) or HT-treated SPI ($n = 12$) for six weeks. The mice were weighed every two weeks. After six weeks of feeding experimental diets, behavioral tests were conducted. Two days after completion of all behavioral tests, all animals were fasted overnight (18 h), and blood was collected from the inferior vena cava using a heparinized syringe under anesthesia with isoflurane. Serum was isolated through centrifugation at $3000 \times g$ for 10 min and stored at -80°C .

For the single administration of HT-treated SPI and non-treated SPI, 7-week-old male C57BL/6J mice were purchased. These mice were given the standard laboratory chow and water ad libitum for five weeks. After overnight fasting for 18 h, the mice were administered 10% (w/v) HT-treated SPI or non-treated SPI in water suspension at 1 g of SPI/kg body weight through sonication. Blood serum was collected in the same manner from the inferior vena cava 30, 60, and 120 min after the administration ($n = 4$ for each group).

Behavioral Tests

Only one behavioral test was conducted per day. The next test was performed after at least two days. Each behavioral test was performed during the light phase, from 9:30 to 14:00. Mice were transferred to

the test room at least 20 min before the test.

The open-field test was performed using an acrylic square box (40 cm in width, 40 cm in length, 30 cm in height). Mice were allowed to freely explore the field for 10 min. The center zone was defined as a 20 cm × 20 cm region in the center of the field. The number of entries into the center zone, time spent in the center zone, and locomotor activity in the whole field were measured using the TopScan LITE system (CleverSys, Reston, VA, USA).

The apparatus of the elevated plus maze test (O'Hara, Tokyo, Japan) consists of two open arms (25 in length, 5 in wide, 0.5 cm in height) and two closed arms (25 × 5 × 16 cm), with a center platform (5 × 5 × 0.5 cm). The plus maze was elevated 50 cm above the floor. In this experiment, a mouse was placed in the center area of the maze with its head directed toward an open arm and allowed to explore the maze freely for 8 min. The number of entries into the open arm, time spent in the open arm, and the distance traveled were measured using the TopScan LITE system.

The object recognition test was performed using the same apparatus as the open-field test. The two sessions, acquisition and recognition, were conducted. A mouse was habituated to the field without objects for 1 h. During the acquisition session, two identical plastic balls (4 cm in diameter) were placed in the field, and the mouse was allowed to explore the objects for 10 min. After the acquisition session, the mouse was removed from the apparatus. The recognition session started 1 h after the acquisition session. Then, one of the objects was replaced with a column-shaped object (3 diameter × 4 cm height), which was considered the novel object, and the remaining ball object was considered the familiar object. The mouse was then placed inside the apparatus and allowed to explore the objects for 8 min. Sniffing within a distance of 1 cm was defined as an approach to the object. The time spent sniffing and the distance traveled in the field were measured using the TopScan LITE system. To measure cognitive performance, a recognition index was calculated by the following equation:

$$\text{Recognition index} = T_{\text{Novel}} / (T_{\text{Novel}} + T_{\text{Familiar}}) \times 100.$$

T_{Novel} : time spent sniffing the novel object, T_{Familiar} : time spent sniffing the familiar object.

The three-chamber social test was performed according to protocols provided by the manufacturer (O'Hara, Tokyo, Japan). The apparatus consisted of three interconnected chambers: left, middle, and right chambers (each size, 20 cm in length × 40 cm in width × 35 cm in height) to allow free access. The left and right chambers had a wire cage to present a mouse. Following habituation to the apparatus without stranger mice for 5 min, the sociability test (session 1) and the social novelty/preference test (session 2) were sequentially conducted. In session 1, a stranger mouse (Stranger 1) was placed in the wire cage in the right chamber, and the subject mouse was allowed to freely explore all three chambers for 10 min. In session 2, Stranger 1 was moved to the wire cage in the left chamber, and a new stranger mouse (Stranger 2) was placed in the right chamber's cage. The subject mouse was again allowed to explore the chambers freely for 10 min. Sniffing within 1 cm in distance was defined as an approach to

the wire cases. The time spent in each chamber and the sniffing time were measured using the TopScan LITE system.

Enzyme-linked Immunosorbent Assay

Serotonin (5-hydroxytryptamine) concentrations in the serum and brain from mice after the behavior test were measured in triplicate using a Serotonin ELISA kit (Abcam, Cambridge, UK) according to the manufacturer's instructions.

Statistical Analysis

All data analyses were performed using GraphPad Prism Version 6.04 (GraphPad Software, San Diego, CA). Food chemical and *in vitro* digestion data are expressed as mean $\pm$ standard deviation, based on three independent preparations ($n = 3$). Animal experiment data, including behavioral tests and serum biochemistry, are expressed as mean $\pm$ standard error of the mean, with biological replicate numbers indicated in the figure legends (typically $n = 12$ per group for behavioral assays). Differences in the average values between two groups (non-treated SPI vs. HT-treated SPI) were analyzed using Student's *t* test or Mann–Whitney *U* test. A *p*-value < 0.05 was considered statistically significant.

Declarations

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Author Contributions

T.T.A.: Integration of all experimental data, funding acquisition, interpretation, investigation, visualization, and writing – original draft. T.M.: Behavioral testing, investigation, funding acquisition, visualization, and writing – original draft. Y.Y.: Food chemistry experiments, investigation, visualization, and writing – original draft preparation. M.Y.: Behavioral testing, investigation, and visualization. H.T.: Supervision and writing – review & editing. N.H.H.: Supervision of behavioral testing and writing – review & editing. S.M.: Preliminary experiments, investigation, visualization, and writing – review & editing. M.N.: Supervision of behavioral testing and writing – review & editing. K.S.: Conceptualization, funding acquisition, overall supervision, and writing – review & editing.

Funding Declaration

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Competing interests

The authors declare no competing financial interests.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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Figures

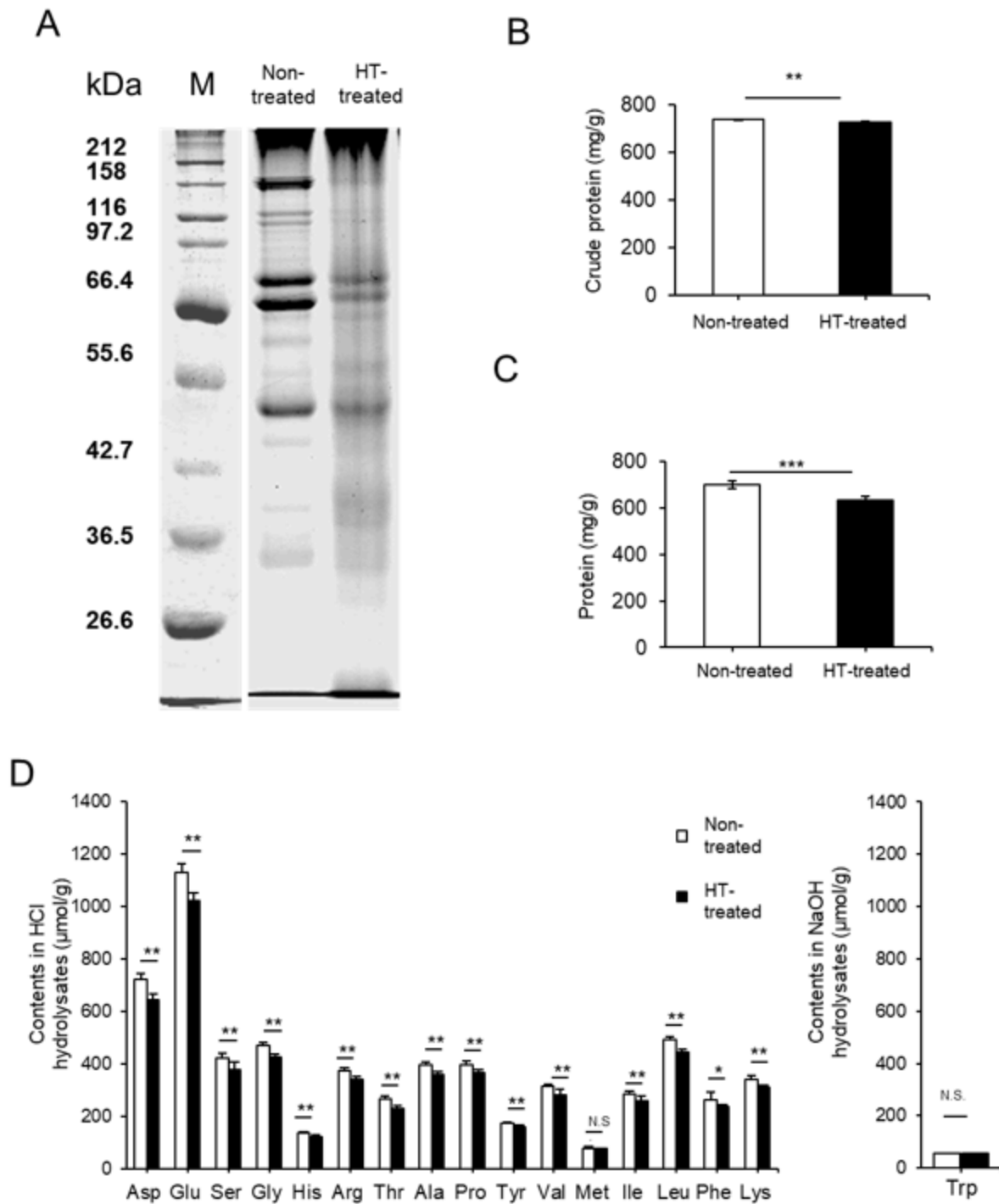


Figure 1

Protein content and amino acid residues in non-treated (□) and HT-treated (■) SPI.

(A) SDS-PAGE profiles of SPI proteins. HT-treated SPI shows diffuse staining and reduced band clarity, indicating protein modification. (B) Crude protein content determined using Kjeldahl analysis. (C) Protein content determined using amino acid analysis. (D) Amino acid content in HCl and NaOH hydrolysates.

Data are presented as mean $\pm$ SD (n = 3–4). P-values were calculated using the unpaired Student's t-test. *p < 0.05, **p < 0.01, ***p < 0.001.

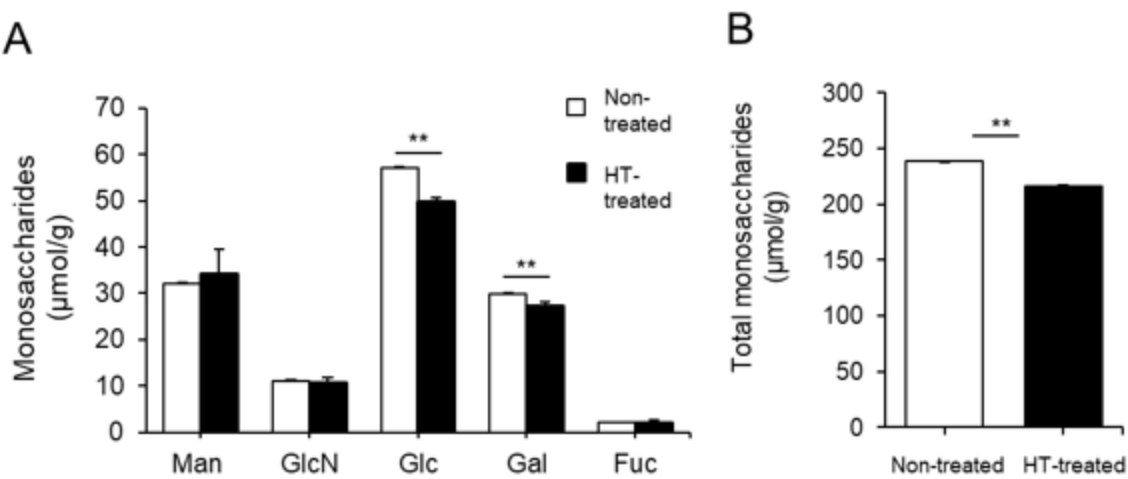


Figure 2
Monosaccharide composition and total sugar content in non-treated (□) and HT-treated (■) SPI.

(A) Individual sugar components quantified using HPLC. (B) Total monosaccharide content. Raffinose-derived sugars were reduced by HT treatment, while glycoprotein-derived sugars remained unchanged. Data are presented as mean $\pm$ SD (n = 4). P-values were calculated using the unpaired Student's t-test. *p < 0.05, **p < 0.01.

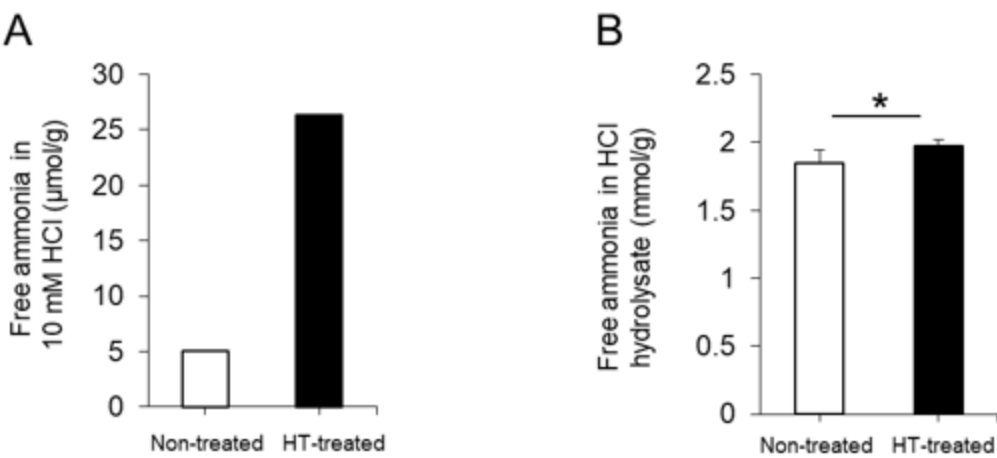


Figure 3
Free ammonia levels in non-treated (□) and HT-treated (■) SPI.

(A) Ammonia levels before HCl hydrolysis (representative value, n = 1). (B) Ammonia levels after HCl hydrolysis (n = 3, mean $\pm$ SD). HT treatment increased ammonia release, suggesting deamidation and side-chain modification. P-values were calculated using the unpaired Student's t-test. *p < 0.05.

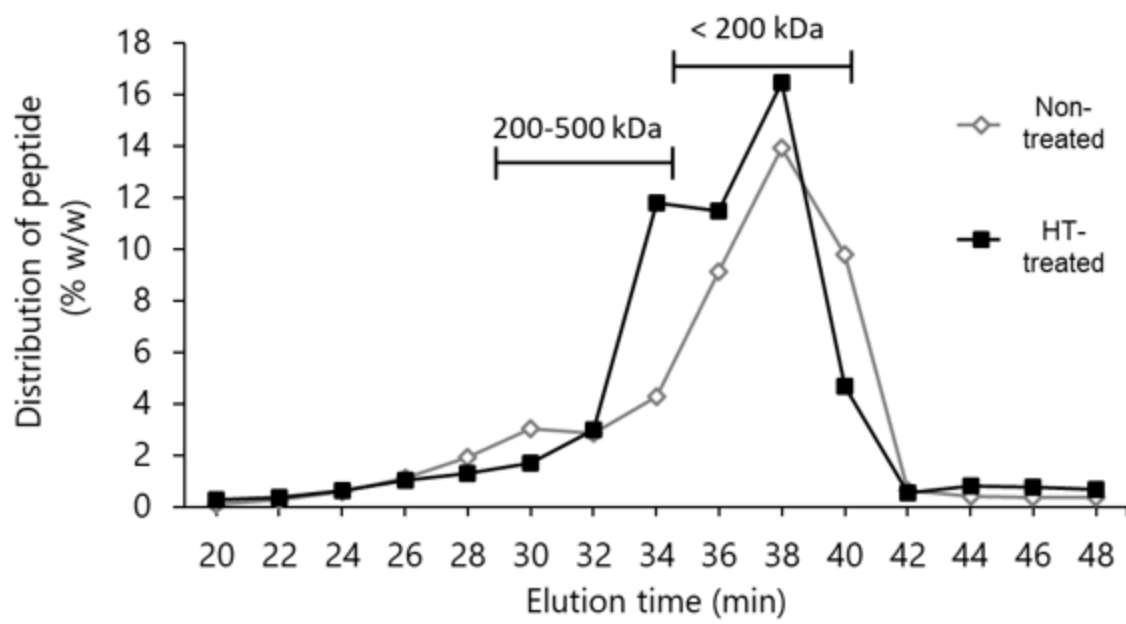


Figure 4

Peptide size distribution in digested SPI analyzed using size exclusion chromatography

Elution profiles of peptides from *in vitro* digests of non-treated (◇) and HT-treated (■) SPI. HT-treated SPI showed increased proportions of peptides in the 200–500 Da range and decreased peptides <200 Da. Molecular weight estimates were based on calibration standards.

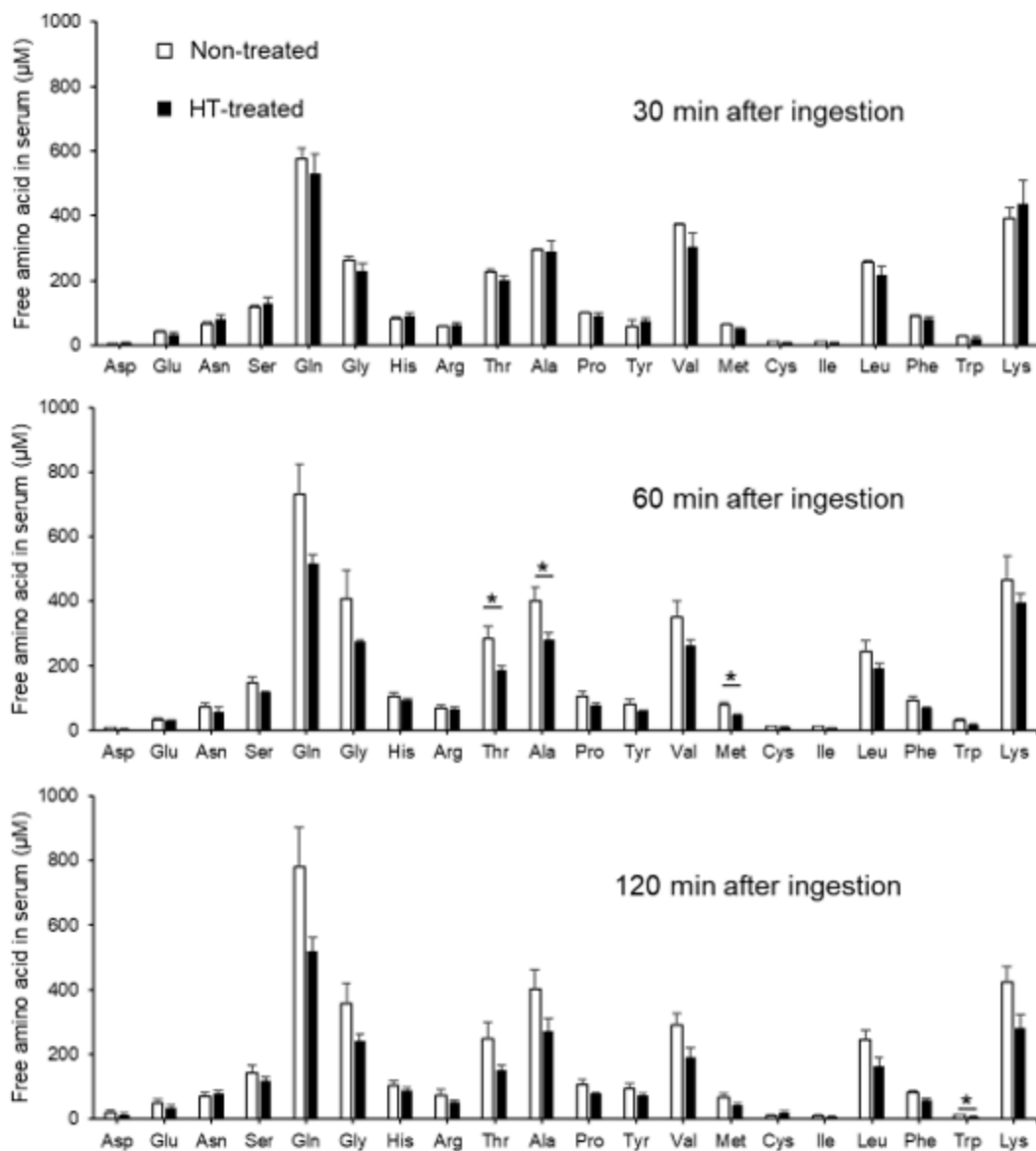
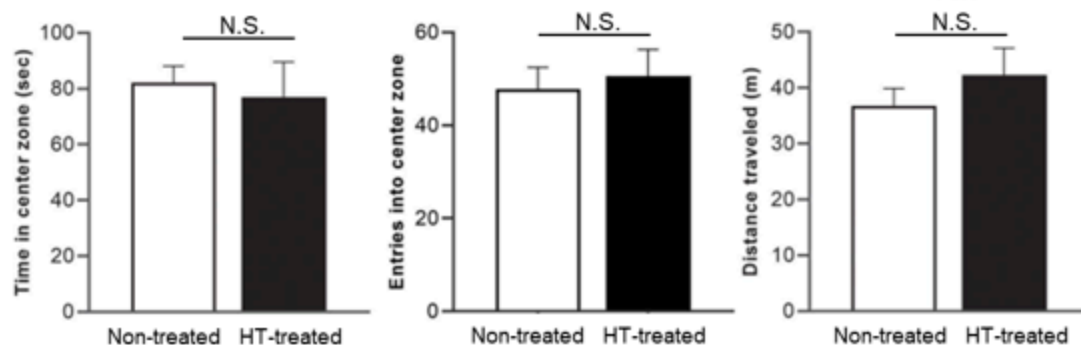


Figure 5

Serum free amino acid levels after single oral administration of SPI

Serum concentrations of free amino acids in mice at 30, 60, and 120 minutes after administration of non-treated (□) or HT-treated (■) SPI. Data are presented as mean $\pm$ SD (n = 4 per group). P-values were calculated using the unpaired Student's t-test. *p < 0.05.

A Open field test



B Elevated plus maze

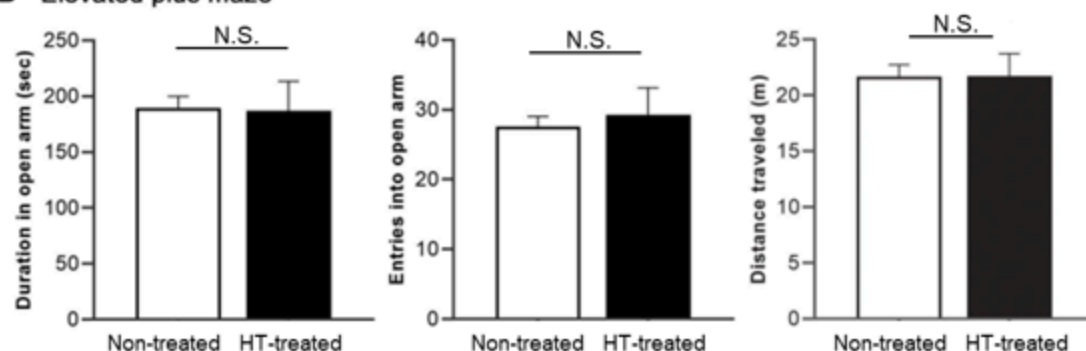


Figure 6

Anxiety-like behavior in mice after long-term feeding of SPI

Behavioral assessment using (A) open-field test and (B) elevated plus maze. No significant differences were observed between non-treated (□) and HT-treated (■) SPI-fed mice. Data are presented as mean ± SEM (n = 12 per group). P-values were calculated using the Mann-Whitney U test. N.S., not significant.

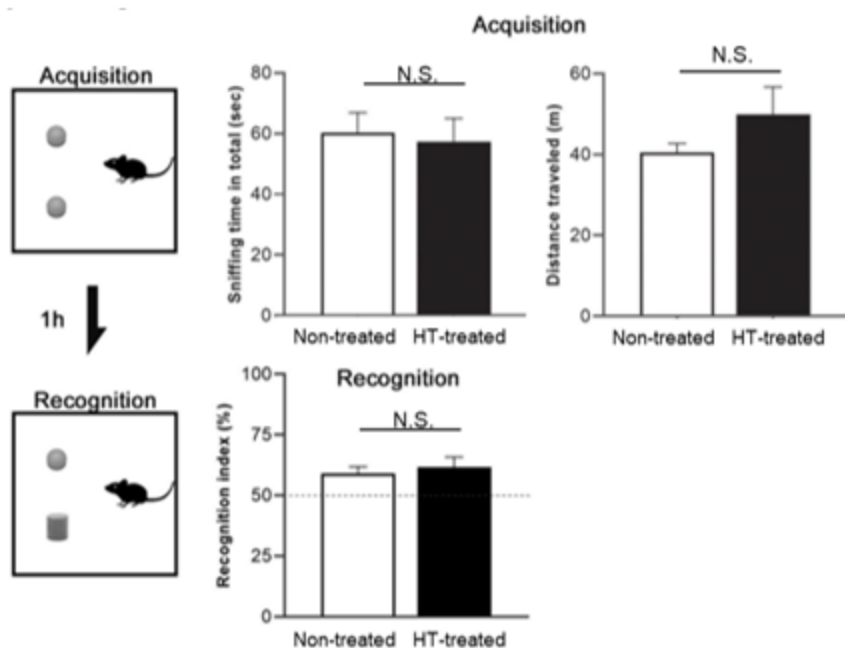


Figure 7

Object recognition memory in mice after long-term SPI feeding

Object recognition test results in mice fed non-treated or HT-treated SPI (n = 8 per group). (Top) Total sniffing time and distance traveled during acquisition. (Bottom) Recognition index calculated 1 h later: $\text{Recognition index} = T_{\text{novel}} / (T_{\text{novel}} + T_{\text{familiar}}) \times 100$. P-values were calculated using the Mann–Whitney U test. N.S., not significant.

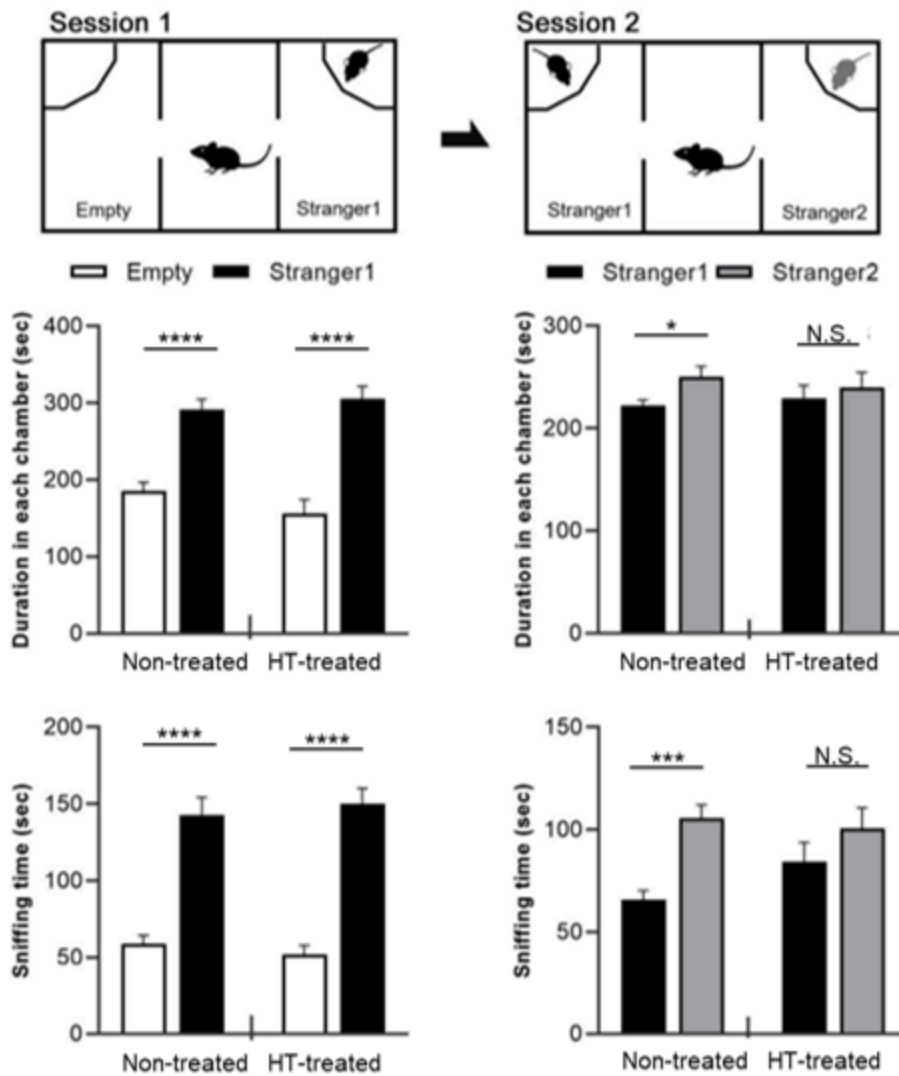


Figure 8

Social behavior in mice after long-term SPI feeding

Three-chamber social test. (Session 1) Mice explored an empty cage (□) or a cage with Stranger 1 (■). (Session 2) Stranger 1 (■) was moved to the opposite chamber, and Stranger 2 (▨) was introduced. Time spent in each chamber (top) and sniffing duration (bottom) were measured. Data are presented as mean ± SEM (n = 12 per group). P-values were calculated using the Mann–Whitney U test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

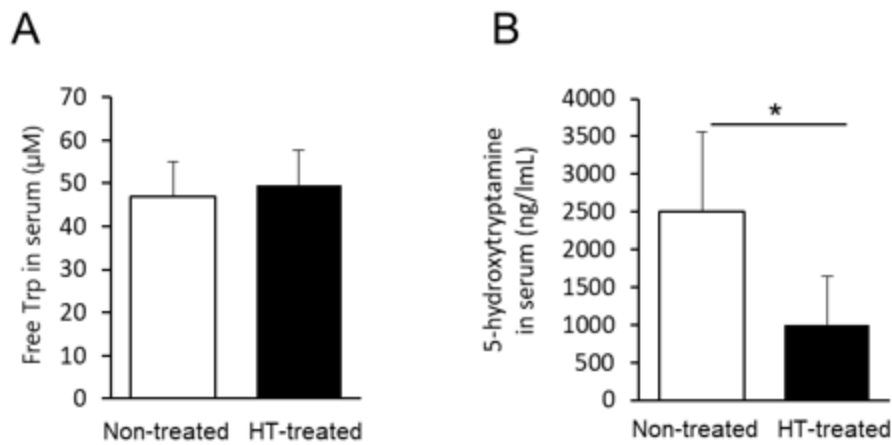


Figure 9

Serum tryptophan and serotonin levels after long-term SPI feeding

Serum concentrations of tryptophan and 5-hydroxytryptamine (serotonin) in mice fed non-treated (□) or HT-treated (■) SPI (n = 7 per group). HT-treated SPI significantly reduced serum serotonin levels, while tryptophan levels remained unaffected. Data are presented as mean ± SEM. P-values were calculated using the Mann–Whitney U test. *p < 0.05.

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