Running title: Potato Extract Suppresses Rat Appetite *via* CCK Secretion

Suppressive Effect on Food Intake of a Potato Extract (Potein®) Involving Cholecystokinin Release in Rats

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Abbreviations: BconB, soybean β-conglycinin bromelain hydrolysate; CCK, cholecystokinin

We have recently reported that oral gavage of a potato extract (Potein®) suppressed the food intake in rats. The satiating effect of the potato extract was compared in the present study to other protein sources, and the involvement of endogenous cholecystokinin (CCK) secretion was examined. Food consumption was measured in 18-hr fasted rats after oral gavage of the potato extract or other protein sources. The CCK-releasing activity of the potato extract was then examined in anesthetized rats with a portal cannula. Oral gavage of the potato extract reduced the food intake in the rats, the effect being greater than with casein and a soybean β-conglycinin hydrolysate. The suppressive effect on appetite of the potato extract was attenuated by treating with a CCK-receptor antagonist (devazepide). The portal CCK concentration was increased after a duodenal administration of the potato extract to anesthetized rats. These results indicate that the potato extract suppressed the food intake in rats through CCK secretion.

Key words: potato extract; cholecystokinin; appetite; rat; devazepide

Obesity is a major cause of metabolic syndrome and is a significant risk factor for the development of diabetes and cardiovascular diseases.[1-3] Obesity rates have increased worldwide, especially in developed countries. This epidemic is primarily due to an excessive supply of food and sedentary work.[3] In addition to daily exercise, appetite control is therefore crucial to avoid over-eating and the development of obesity. Ingested foods

evoke satiety signals in the gut by mechanical stimulation involving gastric expansion or by gut hormonal stimulation, and both of these mechanisms affect the satiety centre *via* afferent nerves.[4,5] The gut hormones with the most robust effects on appetite suppression include cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1) and peptide YY (PYY).[6] Food components that specifically stimulate these satiety-related gut hormones have recently been the focus of nutritional and clinical attempts to reduce over-eating.

The satiety hormone, CCK, is produced by the endocrine I-cells of the gut and also by widespread central and peripheral neurons.[7-9] Dietary protein/peptide directly acts on CCK-producing cells to induce CCK secretion, but also protects endogenous luminal CCK-releasing peptides from tryptic digestion in the intestinal lumen.[10,11] Dietary proteins have been reported to have the most satiating effect among macronutrients,[6] partially due to their potent CCK-releasing activity in the gut.[12-14] Some intact or partially digested proteins (peptone) exert strong stimulatory effects on CCK and other released satiety hormones and may therefore be applicable as functional food components to prevent over-eating.[15] Cuber *et al.* [16] found that peptones, and not whole protein, carbohydrates or fats were strong stimulants of CCK release in the isolated rat duodenojejunum. Cordier-Bussat *et al.*[11] have also proved that peptones directly stimulated CCK secretion in STC-1 cells. It has been found that soybean peptone and pork peptone stimulated the CCK release and suppressed food intake in rats.[17,18]

Potato is one of most satiating foods.[19,20] Potato proteins

contain protease inhibitors, and potato protease inhibitors have been reported to be potentially effective in suppressing carcinogenesis and the appetite.[21] The potato extract, Potein®, is prepared from potato juice which is produced during starch processing. This extract contains concentrated protein fractions and possesses trypsin inhibitory activity. We have recently demonstrated that oral gavage of the potato extract suppressed the food intake in rats.[22] Since the potato extract induced CCK secretion in murine CCK-producing cell line STC-1, it seemed that CCK secretion induced by the potato extract mediated the satiating effect. However, the changes in CCK level and involvement of CCK have yet to be clarified in rats. We compared in the present study the effect on rat appetite of the potato extract to that of other protein sources, and examined the involvement of CCK in this process.

Materials and Methods

Materials. The potato extract (Potein®) was prepared by concentrating and filtering potato juice (a by-product of potato starch processing), and was supplied by Toyo Shinyaku Co. (Fukuoka, Japan). It consisted of 19.9% protein, 59.8% carbohydrate, 4.2% fibre, 0.2% fat, 11.8% ash and 4.1% water, this being analyzed by Japan Food Research Laboratories in Tokyo, Japan. The soybean β-conglycinin bromelain hydrolysate (BconB)[23] was kindly provided by Fuji Oil Co. (Osaka, Japan), and the devazepide (L364,718) was from ML Laboratories (Liverpool, UK). All other materials were purchased from Wako (Osaka, Japan) unless otherwise specified.

Animal feeding experiments (Experiments 1-3). Male Sprague Dawley rats were purchased from Japan SLC (Hamamatsu, Japan) and were fed an AIN-93G-based semi-purified diet.[24] This diet consisted of 250 g/kg of casein, 602.5 g/kg of sucrose, 50 g/kg of soybean oil, 50 g/kg of cellulose, 35 g/kg of a mineral mixture (AIN-93G), 10 g/kg of a vitamin mixture (AIN-93G), and 2.5 g/kg of choline bitartrate. Each rat was housed individually in a temperature- and humidity-controlled room (22 ± 2°C, 55 ± 5%) under a 12:12-h light-dark cycle (lights on from 02:00–14:00). To observe clear changes in the food consumption, and based on our preliminary studies, the rats had access to the food diet for 6 h during the dark period (14:00–20:00) after 18 h of fasting. The rats were trained daily for the intra-gastric administration with water by a feeding tube (Safeed feeding tube Fr.5, 40 cm; Terumo, Tokyo, Japan) before being given the diet. After the animals had acclimatised for 7 d until the daily food intake had become stable, the rats received a test solution through the feeding tube. Feeding experiments were conducted by using a crossover study design which was carried out until each rat had received all of the treatments. Water (6 mL/kg BW) was orally administered in all experiments as a negative control. The food consumption was measured 1, 2, 3 and 6 h after the oral administration of the test samples and diet feeding.

This study was approved by the Hokkaido University Animal Committee, and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals at Hokkaido University.

We investigated in experiment 1 the dose response to the potato extract (1.0 and 1.5 g/kg BW) on the food intake. Experiment 2 was designed to compare the effect of the potato extract on the food intake with other protein sources. The potato extract, casein sodium or BconB was orally administered at a dose of 1.0 g/kg BW. We had found from our previous study that BconB had an appetite-suppressing effect on rats under meal-feeding conditions.[23] Experiment 3 was conducted to examine the involvement of CCK secretion in the suppressive effect of the potato extract on the food intake. A solution containing a CCK-A receptor antagonist (devazepide at 500 μg/kg BW) or vehicle (10% DMSO and 10% TWEEN-80 in sterilised saline) was intraperitoneally injected (1000 μL/kg BW) before the oral administration of the potato extract (1.0 g/kg BW) or water (6 mL/kg BW), and the food intake was measured as just described.

In situ CCK secretion experiment (Experiment 4). The *in situ* experiment was carried out similarly to our previous study with some modifications.[25] Briefly, the small tip (7–8 mm) of an SP 28 polyethylene catheter (0.4 mm ID, 0.8 mm OD; Natsume Seisakusyo, Tokyo, Japan) connected to a Silascon 00 silicone catheter (0.5 mm ID, 1.0 mm OD; Kaneka, Osaka, Japan) was inserted into the portal vein under anaesthesia with ketamine (80 mg/kg BW; Ketaral, Daiichi Sankyo, Tokyo, Japan) mixed with xylazine (12 mg/kg BW, MP Biomedicals, Irvine, CA, USA). The pylorus was ligated to avoid any backflow of the test solution into the stomach. Blood samples (300 μL) for CCK and gastrin measurements were withdrawn into a syringe containing EDTA

(1 mg/mL final concentration) and aprotinin (0.6 TIU/mL final concentration) through the portal catheter. After collecting the basal (0 min) blood sample, deionized water (6 mL/kg as a negative control) or the potato extract solution (0.5 g/kg) was directly administered into the duodenum. Because the direct injection of the potato extract into the duodenum without passing through the stomach, the dose used in this experiment was lower (half the dose as that for oral gavage) than that used for oral administration. Portal blood was collected through the catheter 15, 30, 60, 90, and 120 min after the duodenal administration. The catheter was filled with saline containing heparin (50 IU/mL final concentration; Ajinomoto, Tokyo, Japan) between each blood collection. Additional anaesthetic (ketamine mixed with xylazine) was injected during the experiment to keep the rats anaesthetised, and the rat body temperature was maintained with a heating pad. Plasma samples were separated from the blood and extracted by using ethanol as previously described for CCK measurements.[26] The plasma CCK concentrations were measured with an EIA kit (Phoenix Pharmaceuticals, Belmont, CA, USA). The primary antiserum cross-reacts 100% with sulphated and non-sulphated CCK (26-33), CCK-33 (porcine), CCK (27-33), caerulein, gastrin-1 (human) and big gastrin-1 (human), and this antiserum cross-reacts 12.8% with CCK (30-33). The coefficients of intra- and inter-assay variation were within 5-10% and <15%, respectively.

To determine whether the changes in concentration measured by EIA reflected the absolute changes in CCK or gastrin, we also measured the gastrin concentration in identical plasma samples (0

and 90 min) collected from the potato extract-administered rats; we used a gastrin ELISA kit (Assay Designs, Ann Arbor, MI, USA) which has low cross-reactivity with CCK. The primary antibody in the gastrin ELISA kit cross-reacts 100% with gastrin I (G17-1), 74.6% with minigastrin (G13- I), 70.7% with gastrin I (rat), 9.3% with gastrin II (G17-II, sulphated), 2.67% with cholecystokinin 26-33 (CCK-8), 1.6% with gastrin tetrapeptide (CCK-4), 0.8% with big gastrin (G34-I) and <0.001% with other gut hormones. The coefficients of intra- and inter-assay variation were <9% and <7%, respectively.

CCK secretion in the STC-1 cell culture (Experiment 5). STC-1 cells (presented by Dr. D. Hanahan, University of California, San Francisco, CA, USA) were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum, 50 IU/mL of penicillin and 500 μg/mL of streptomycin in a humidified 5% CO_2 atmosphere at 37°C. The cells were routinely subcultured by trypsinisation upon reaching 80–90% confluency. The cells were grown in 48-well culture plates at a density of 1.25×10^5 cells/well for 2–3 d until they had reached 80–90% confluency. The cells were washed three times with a Hepes buffer to remove the culture medium and then exposed to 100 μL of the test agent (5 mg/mL of the potato extract or BconB dissolved in the same buffer) for 60 min (37°C in 5% CO_2). The Hepes buffer was composed of 140 mM NaCl, 4.5 mM KCl, 20 mM Hepes, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D-glucose and 0.1% BSA (pH 7.4). After incubating for 60 min, the supernatants from the 48-well culture plates were collected and

centrifuged at 800 × g for 5 min at 4°C to remove the remaining cells and then stored at below −50°C until being used to measure the CCK concentration by EIA as already described.

Statistical analysis. Results are presented as the mean ± SEM. Statistical significance was assessed by Dunnett's- or unpaired t-test after one-way or two-way ANOVA, as described in figure legends. $p < 0.05$ was considered statistically significant.

Results

Food intake was measured 1, 2, 3 and 6 h after the orogastric administration of different doses of the potato extract to rats that had been fasted for 18 h. The food intake in rats that had been preloaded with 1.5 kg/kg of the potato extract tended to be lower than that in rats control preloaded from 1 h to 3 h, this difference being statistically significant 6 h after the administration ($p < 0.05$ by Dunnett's test) (Fig. 1). A lower dose of the potato extract (1.0 g/kg) had a similar effect to the 1.5 g/kg dose.

Although the absolute food intake in the potato extract-treated rats appeared to be lower than the other group in experiment 2, no statistically significant differences were apparent by one-way ANOVA and the post-hoc test (Fig. 2A). However, relative food intake in the potato extract-treated rats was significantly lower than that in the control treatment 1, 2, 3 and 6 h after administration (Fig. 2B). The relative food intake in the casein- or BconB-treated rats was significantly lower only by the first 1 h after oral administration. The extent of this lower food intake appeared to be greatest with the potato extract-treated rats than with the rats given the other treatments at each time.

Prior to orogastric administration of the potato extract, the rats intraperitoneally (*i.p.*) received 500 μg/kg of the CCK receptor antagonist, devazepide, or a vehicle. Figure 3 shows that preloading the potato extract significantly decreased the subsequent food intake 2 and 3 h after administration when compared to water preloading of the vehicle-treated rats. However, when the rats were treated with devazepide, no significant reduction was apparent after administering the potato extract. The devazepide treatment showed no marked effect on the food intake in the water-preloaded rats.

To examine the effect of the potato extract on CCK secretion in the rat intestines, the potato extract was directly administered into the duodenum of anaesthetised rats. The CCK-EIA kit cross-reacts with gastrin according to the manufacturer. Any backflow of the test solutions into the stomach in our experiment was prevented by closing the pylorus, and gastrin was not secreted from the small intestine. Changes in the CCK level from the basal state (ΔCCK) could therefore be considered as CCK fluctuations induced by the treatment. The basal CCK levels were 45.7 ± 8.7 pM in the control group and 56.1 ± 9.2 pM in the potato extract-treated group (p = 0.429). The plasma CCK concentration gradually increased following the administration of the potato extract (Fig. 4A). The increment of CCK peaked at 90 min and was significantly higher than the basal value (0 min). The CCK concentration did not significantly change in the control group. The AUC value of the increased CCK level during 120 min (ΔAUC) after duodenal administration of the potato extract was significantly greater than that after water administration (Fig. 4B).

The portal gastrin level was not increased after duodenal administration of the potato extract (135.2 ± 10.9 pM at 0 min and 157.5 ± 20.5 pM at 90 min, $p = 0.375$). This indicates that the increment in measured CCK was not due to the increment from cross-reacting gastrin.

To examine the direct effect of the potato extract on CCK-producing enteroendocrine cells, STC-1 cells were exposed to the potato extract solution for 1 h. BconB was used as a positive control, as we had previously demonstrated its CCK-releasing activity in STC-1 cells.[23] Both the potato extract and BconB treatment induced significant increases in the CCK concentration of the supernatant of STC-1 cells when compared to the control treatment (Fig. 5).

Discussion

The results of the present study suggest that oral administration of the potato extract suppressed the appetite of the rats through a CCK-dependent mechanism. *In vivo* experiments showed that oral gavage with 1.0 g/kg of the potato extract significantly decreased the food intake during the 6 hours after its administration (Figs. 1 and 2B). When the effect of the potato extract was compared with that of other dietary proteins/peptides (casein and BconB) at the same dose, the relative food intake markedly showed that the potato extract suppressed the food intake to a greater extent (Fig. 2B). This indicates that the effect of administering the potato extract was not simply mediated by preloading with protein/peptide as an energy source. We have previously demonstrated the CCK-mediated suppressive effect of a

soybean ß-conglycinin hydrolysate on the food intake in rats.[17,23] Although the experimental conditions in this study were different from those in previous studies, the results shown in Fig. 2 demonstrate a similar or even slightly greater potency of the potato extract when compared to BconB in suppressing the food intake.

The reduction in food intake caused by potato extract preloading was attenuated by the devazepide treatment, as shown in Fig. 3. Devazepide, also known as MK-329 or L-364,718, is a specific antagonist for the type-A CCK receptor, and it can attenuate the inhibitory effect of various nutrients on the food intake in rats.[27-30] The result from experiment 3 strongly suggests that CCK was involved in suppressing the food intake mediated by the potato extract administration.

The portal CCK concentration was significantly increased by duodenal administration of the potato extract to anaesthetised rats (Fig. 4). This result supports the notion that the suppressive effect of the potato extract on food intake involved CCK secretion. The portal CCK concentration gradually increased up to 90 min after duodenal administration of the potato extract. Although the experiment was performed under anaesthesia, the gradual increase in portal CCK could be the one of the reasons why the potato extract suppressed the food intake for such a long time (Figs. 1-3).

Similarly to BconB, the potato extract significantly stimulated CCK release in the STC-1 enteroendocrine cell line (Fig. 5), confirming that the potato extract could act directly on CCK-producing enteroendocrine cells to trigger CCK secretion independently of the trypsin inhibitory activity. Several studies have shown that intact proteins or their hydrolysates could

stimulate the secretion of CCK from enteroendocrine cells,[11,15,31] implying that certain protein-related components of the potato extract directly affected the CCK-producing cells.

It has recently been reported that a protease inhibitor derived from the potato reduced the food intake by increasing the CCK level in rats.[32] Since the protease inhibitor failed to stimulate CCK secretion in STC-1 cells, it was concluded that the effect depended on luminal trypsin inhibition, and not on its direct action on CCK cells. In contrast, we observed that the potato extract directly stimulated CCK secretion from STC-1 cells (Fig. 5), consistent with the findings in a recent report.[22] The potato extract included proteins other than trypsin inhibitors and other non-protein components, so some of these components might be responsible for the direct stimulation of CCK secretion. It is likely that CCK secretion is the result of both the direct action of an active compound on CCK-producing cells and luminal protease inhibition *in vivo*. The major components of the potato extract include carbohydrate and proteins; however, there are few reports to support any effect of carbohydrates on CCK secretion. It would be interesting if not only proteins or peptides, but also oligosaccharides or such other minor phytochemicals as flavonoids had the ability to directly stimulate CCK secretion. Our previous study has shown that *in vitro* digestion by pepsin and/or pancreatin enhanced the effect of the potato extract on CCK secretion in STC-1 cells, suggesting that peptides from the potato extract liberated by the luminal digestion were responsible for stimulating this CCK release.[22] Further studies are necessary to identify the active components of the potato extract that directly induce CCK

secretion and the molecular mechanisms involved in this process.

The relatively delayed peak (90 min) of CCK secretion in the *in situ* experiment (Fig. 4A) might be due to the slower liberation of active peptides from the potato extract by its trypsin inhibitory activity. The trypsin inhibitory activity of the potato extract may also be responsible for sustaining (3-6 h) the suppression of food intake *in vivo* (Figs. 1-3), because inhibition by luminal trypsin slows the digestion of ingested proteins, resulting in the continuous stimulation of CCK secretion by luminal dietary protein/peptides. It would be interesting to examine whether other satiating hormones such as GLP-1 and PYY released from the distal small intestine are involved in the effect of the potato extract. In addition, the slower digestion of carbohydrates due to the indigestible saccharides contained in the potato extract might have affected GLP-1/PYY secretion to enhance the satiating effect.

We only investigated in the present study the acute effects of administering the potato extract on the food intake in rats, and it is important to know whether chronic ingestion of the potato extract would affect the daily food intake and subsequent body weight gain. There is another possibility that the potato extract would attenuate post-prandial hyperglycemia through the inhibition of gastric emptying due to enhanced CCK secretion. Such studies on humans would provide evidence as to whether the potato extract could be a novel functional food for preventing obesity and lifestyle-related diseases.

In summary, oral preloading with the potato extract reduced the subsequent food intake in rats, and this inhibitory effect was abolished after treating with a CCK-A receptor antagonist. The

portal CCK level was significantly elevated following luminal administration of the potato extract. Together with the direct stimulation of CCK secretion in enteroendocrine STC-1 cells by the potato extract, we conclude that the potato extract suppressed the rat appetite *via* enhanced CCK secretion.

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Fig. 1. Effect of Orogastric Administration of the Potato Extract on the Subsequent Food Intake of Fasted Rats.

The accumulated food consumption was measured 1, 2, 3 and 6 h after the orogastric administration of 6 mL/kg of water as a control (unfilled bars), 1.0 g/kg (hatched bars) or 1.5 g/kg (filled bars) of the potato extract. Results are expressed as the mean ± SEM (n=12). The one-way ANOVA *p* values are 0.273 at 1 h, 0.181 at 2 h, 0.123 at 3 h, and 0.023 at 6 h. Asterisks (*) indicate significant differences between the treated and control (water) groups at the same time point ($p < 0.05$, Dunnett's test).

Fig. 2. Comparison of Preloading Various Protein and Potato Extracts on the Food Intake in Fasted Rats.

The test preparations (unfilled bars, water; hatched bars, casein; cross-hatched bars, BconB; filled bars, the potato extract) were orally administered at a dose of 1.0 g/kg BW. The absolute food intake (A) and relative food intake to the control group considered as 100% (B) are presented. Results are expressed as the mean ± SEM (n=7). The one-way ANOVA *p* values of the absolute intake (A) are 0.163 at 1 h, 0.115 at 2 h, 0.266 at 3 h, and 0.115 at 6 h. The one-way ANOVA *p* values of the relative intake (B) are 0.006 at 1 h, 0.006 at 2 h, 0.031 at 3 h, and 0.048 at 6 h. Asterisks (*) represent significant differences between the treated and control (water)

groups at the same time point ($p < 0.05$, Dunnett's test).

Fig. 3. Effect of the CCK-A Receptor Antagonist on potato Extract-Mediated Reduction of the Food Intake.

Devazepide (500 µg/kg BW) or a vehicle was intraperitoneally (*i.p.*) injected immediately prior to orogastric administration of the potato extract (1.0 g/kg BW). Unfilled bars, vehicle + water; hatched bars, vehicle + 1.0 g/kg of the potato extract; cross-hatched bars, 500 µg/kg of devazepide + water; filled bars, 500 µg/kg of devazepide + 1.0 g/kg of the potato extract. Results are expressed as the mean ± SEM (n=20). The one-way ANOVA *p* values are 0.189 at 1 h, 0.038 at 2 h, 0.009 at 3 h, and 0.222 at 6 h. Asterisks (*) indicate significant differences between the treated and control (vehicle + water) groups at the same time point ($p < 0.05$, Dunnett's test).

Fig. 4. Effect of Duodenal Administration of the Potato Extract on the Portal CCK Concentration in Anaesthetised Rats.

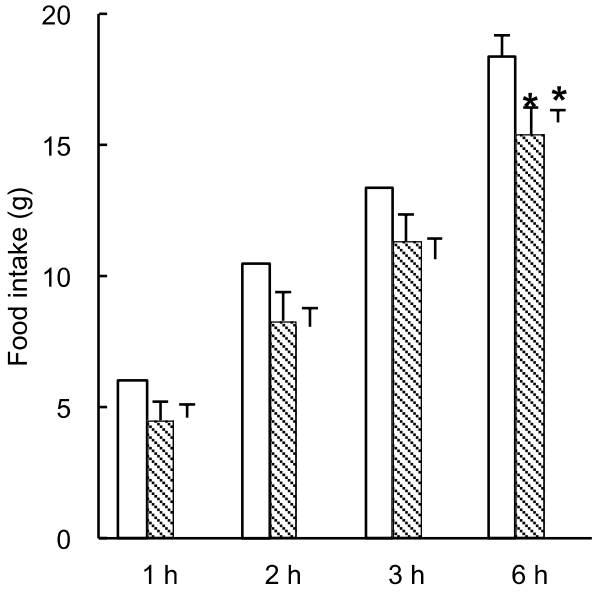
Portal blood was collected before (0 min) and after (15, 30, 60, 90 and 120 min) administration of the potato extract (filled circles, 0.5 g/kg BW) or water (unfilled circles, 6 mL/kg BW) into the duodenum. The change in plasma CCK

concentration from the basal level (A) and the area under the curve of the change in CCK (ΔAUC) (B) are presented. Results are expressed as the mean ± SEM (n=7). The respective two-way ANOVA *p* values are 0.063, < 0.001, 0.046 for the treatment, time, and treatment × time. A, The plus sign (+) represents significant difference between the basal (0 min) and the designated time point in each group ($p < 0.05$, Dunnett's test). B, The asterisk (*) represents significant difference between the potato extract group and the water group ($p < 0.05$, *t*-test).

Fig. 5. Effect of the Potato Extract and BconB on CCK Secretion in STC-1 Cells.

The CCK levels were measured in the supernatants of STC-1 cells after exposure to the test preparations at 5 mg/mL for 1 h. Data are expressed as the mean ± SEM of 4 repeated experiments. The one-way ANOVA *p* value is < 0.001. Asterisks (*) indicate significant differences compared to the control treatment ($p < 0.05$, Dunnett's test).

1 Figure 1

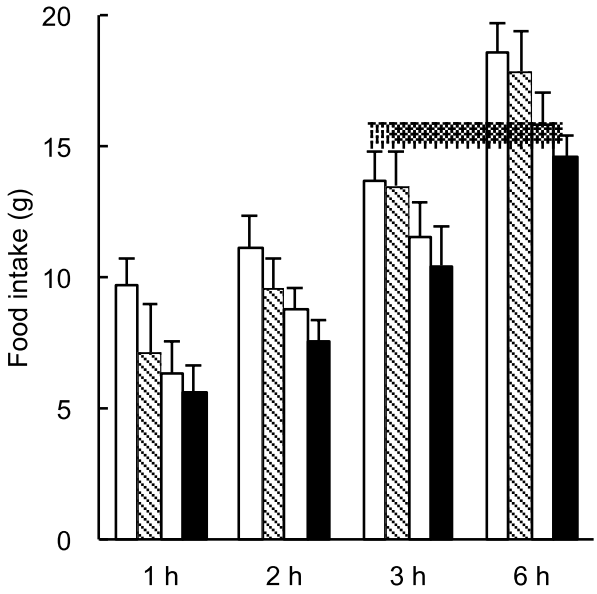


2

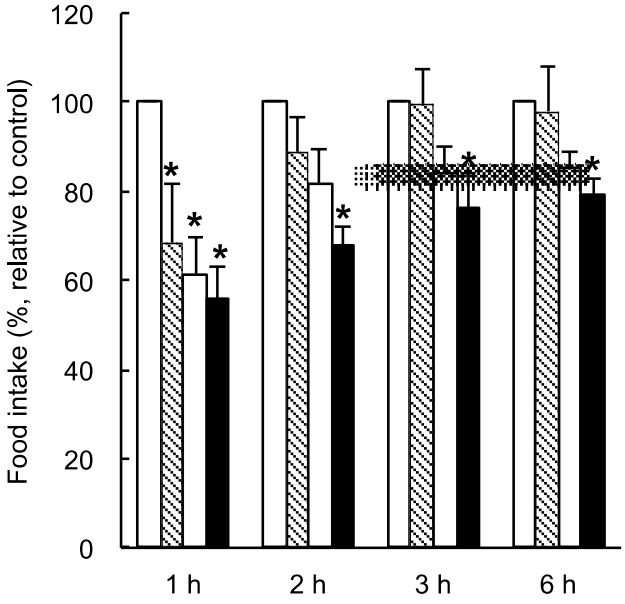
3

Figure 2

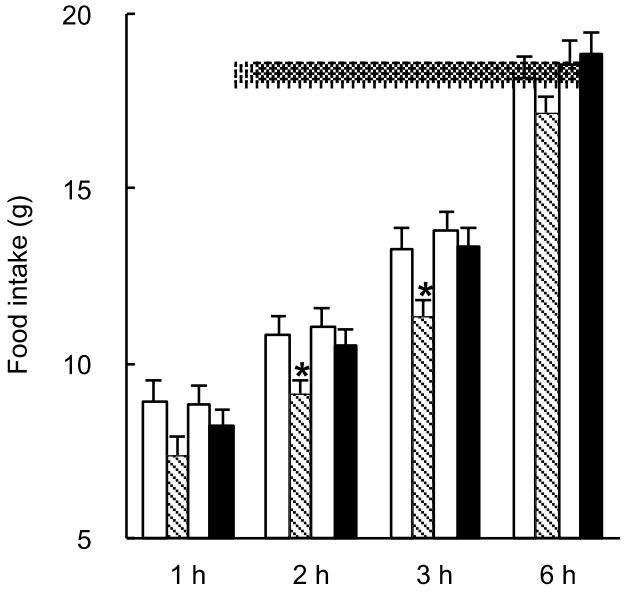
A


20
15
10
5
0
Food intake (g)
1 h
2 h
3 h
6 h

B


120
100
80
60
40
20
0
Food intake (%, relative to control)
*
1 h
2 h
3 h
6 h

1 Figure 3

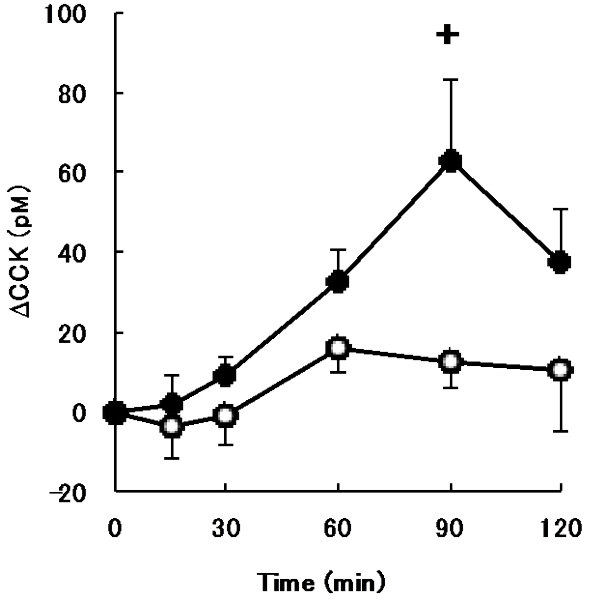


2

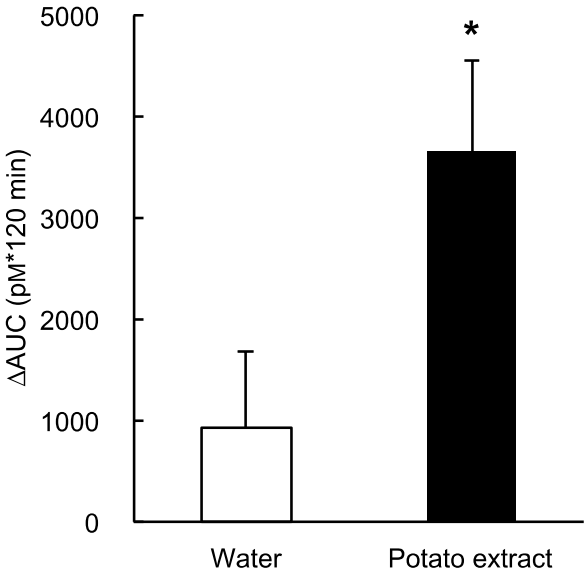
3

Figure 4

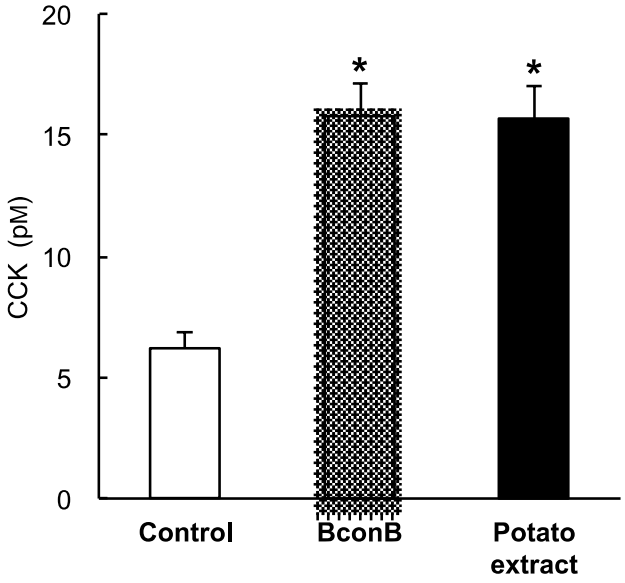
A


100
80
60
40
20
0
-20
ΔCCK (pM)
+
0
30
60
90
120
Time (min)

B


5000
4000
3000
2000
1000
0
ΔAUC (pM*120 min)
*
Water
Potato extract

1 Figure 5



2