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Sufficient intake of high amylose cornstarch maintains high colonic hydrogen production for 24 h in rats

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Colonic hydrogen (H_2) can suppress oxidative stress and damage in the body. We examined the minimum requirement of high amylose cornstarch (HAS) to maintain high colonic H_2 production for 24 h. Ileorectostomized and sham-operated rats were fed a control diet supplemented with or without 20% HAS for 7 days. Colonic starch utilization was determined. Next, rats were fed the control diet with or without 10% or 20% HAS for 14 or 28 days, respectively. Breath and flatus H_2 excretion for 24 h was measured. 1.04 g of resistant fraction in HAS was utilized for 24 h by colonic bacteria. High H_2 excretion was not maintained for 24 h in rats fed the 10% HAS diet, from which only 0.89 g of resistant starch was estimated to be delivered. High colonic H_2 production for 24 h would be maintained by delivering more HAS to the large intestine than is utilized.

Key words: hydrogen; high amylose cornstarch; resistant starch; colonic fermentation; rats

Excessive oxidative stress can trigger the onset and progression of various metabolic syndromes such as diabetes mellitus, hypertension, obesity, dyslipidemia, and pro-inflammatory status.[1,2] Many studies have demonstrated that providing a supply of molecular hydrogen (H_2) by H_2 gas or H_2 water alleviated oxidative lesions in various animal models[3–6] since Ohsawa et al. reported that oxidative stress and damage due to brain ischemia-reperfusion were relieved by inhaling H_2 gas[6]. We found that H_2 produced by colonic fermentation of high amylose cornstarch (HAS), which is a non-digestible saccharide, suppressed oxidative stress and damage in hepatic ischemia-reperfusion rats.[7] Also, in our previous study, it was demonstrated that colonic H_2 passes through the colonic wall into the abdominal cavity and that H_2 decreased the abundance of adipose *Il-6* mRNA.[8] Therefore, colonic H_2 would be a potent antioxidant in the body.

Although inhalation of H_2 gas can deliver a large amount of H_2 into the body, it is difficult to use the tool daily as an H_2 supplier because of the necessity of equipment. Administration of H_2 water cannot deliver a large amount of H_2 due to the low solubility of H_2 in water. Unlike H_2 gas and H_2 water, colonic H_2 can be continuously delivered in the body without appropriate equipment if a fermentation substrate is supplied. Therefore, colonic H_2 would be more available for daily delivery of H_2 than H_2 gas and H_2 water. High H_2 production for 24 h could contribute to the control of oxidative stress. However, it remains unclear whether a high level of colonic H_2 is produced for 24 h upon ingesting a fermentation substrate.

H_2 excretion in breath and flatus is a good indicator reflecting colonic H_2 production. Many investigators have examined the change in breath H_2 excretion in humans who had been administered non-digestible saccharides in order to measure the orocecal transit time[9,10] and diagnose lactose intolerance.[11,12] Some studies demonstrated increased H_2 excretion by feeding non-digestible saccharides such as dietary fiber, resistant starch, and oligosaccharides in rats.[13,14] However, to our knowledge, changes in H_2 excretion over 24 h were not confirmed in rats. It is indispensable to accurately determine the amount of non-digestible saccharides that needs to be ingested so that a high level of colonic H_2 is continuously produced for 24 h in order to examine the antioxidative effect of colonic H_2. Although breath H_2 excretion increased after a single administration of 10 g of lactulose or 20–30 g of bran in human, high H_2 excretion was not maintained for 24 h.[15] On the other hand, according to the studies of Muir et al.[16] and van Munster et al.,[17] relatively high H_2 excretion in breath was maintained for 24 h in humans when they were administered resistant starch with 3 meals a day. Therefore, it also remains unclear whether high colonic H_2 production is sustained by some non-digestible saccharides.

In the present study, we examined whether high production of colonic H_2 was maintained for 24 h in rats fed HAS and how much HAS needs to be ingested to

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Abbreviations: H_2, hydrogen; HAS, high amylose cornstarch; ZT, Zeitgeber time.

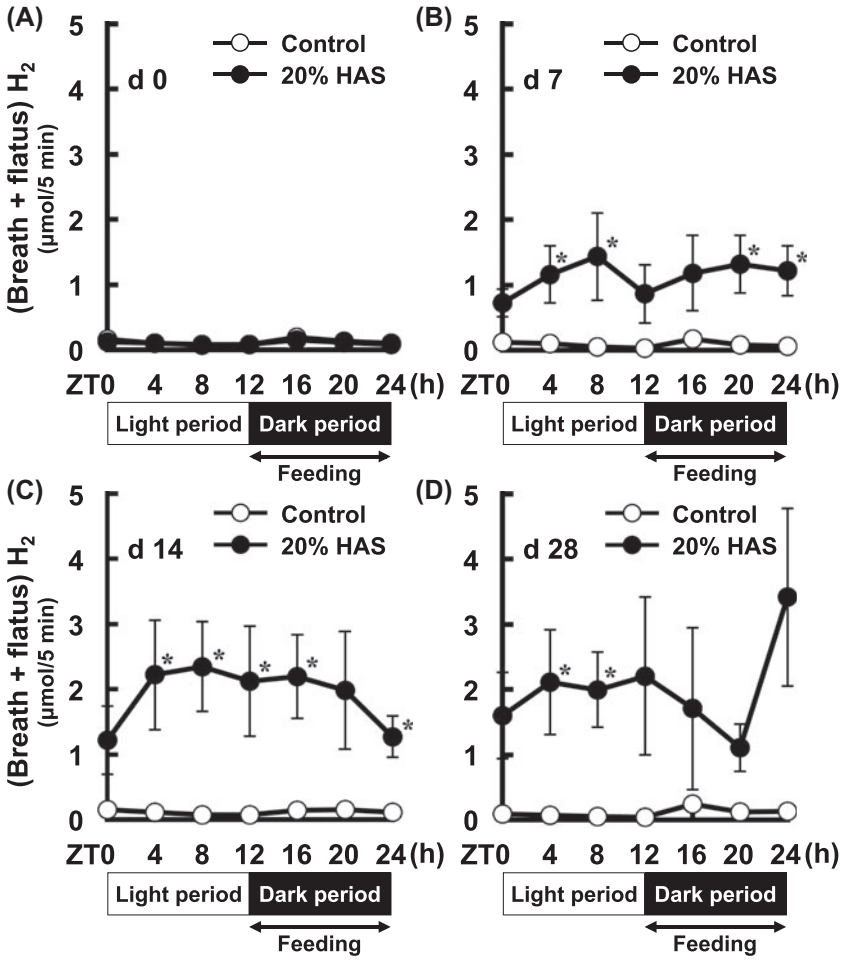

Fig. 1. Time course of hydrogen (H_2) excretion in breath and flatus in rats fed the 20% high amylose cornstarch diet over a 24-h period on d 0, 7, 14, and 28. (A) d 0, (B) d 7, (C) d 14, and (D) d 28.

Notes: Values are means with their standard errors represented by vertical bars, $n = 9$. Mean values indicated by single asterisk were significantly different compared with the control group ($p < 0.05$). Repeated-measures, 2-factor ANOVA was used to analyze H_2 excretion between the control and 20% HAS groups across time: HAS, $p = 0.3625$; time, $p = 0.0131$; interaction, $p = 0.9780$ at d 0; HAS, $p < 0.0001$; time, $p = 0.9431$; interaction, $p = 0.9361$ at d 7; HAS, $p < 0.0001$; time, $p = 0.8717$; interaction, $p = 0.8310$ at d 14; HAS, $p < 0.0001$; time, $p = 0.7623$; interaction, $p = 0.7461$ at d 28. 20% HAS, rats fed the control diet supplemented with 20% high amylose cornstarch; ZT, Zeitgeber time.

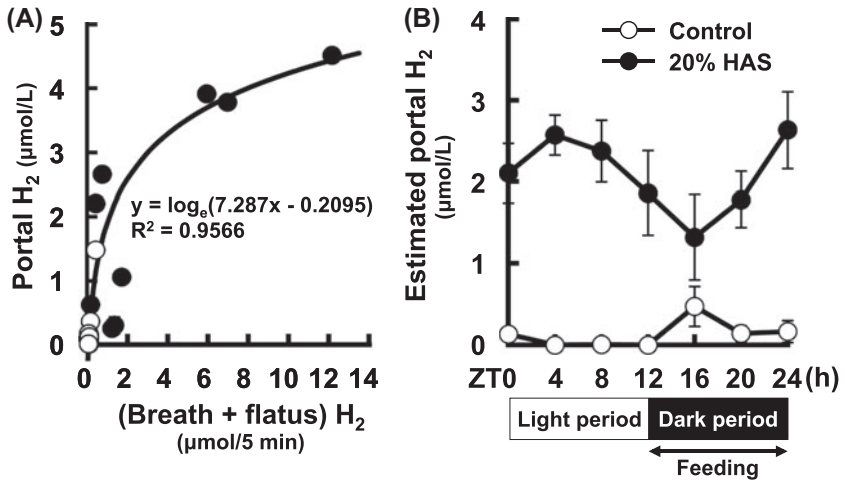

Fig. 2. (A) Correlation between portal H_2 concentration and breath and flatus H_2 excretion and (B) change in estimated portal H_2 concentration over a 24-h period in rats fed the 20% high amylose cornstarch diet.

Notes: (A) Each point is the value of individual rats (n 18). Open circles are the value of rats fed the control diet and closed circles are the value of those fed the 20% HAS diet. The line indicates the relationship between portal H_2 concentration and H_2 excretion in breath and flatus: $Y = \log_e(7.287X - 0.2095)$, $R^2 = 0.9566$. Y is the portal H_2 concentration and X the H_2 excretion in breath and flatus. (B) Values are means with their standard errors represented by vertical bars, ($n = 9$). 20% HAS, rats fed the control diet supplemented with 20% high amylose cornstarch; ZT, Zeitgeber time.

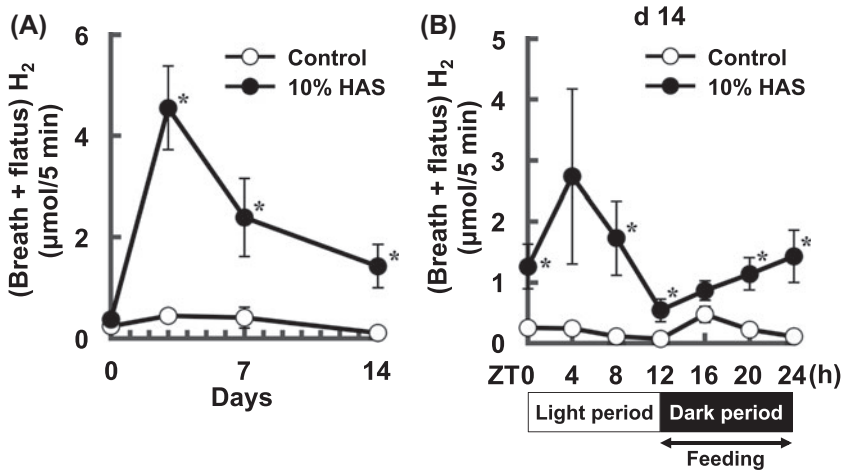

Fig. 3. Changes in breath and flatus hydrogen (H_2) excretion in rats fed the 10% HAS diet (A) for 14 days and (B) over a 24-h period on d 14.
Notes: Values are means with their standard errors represented by vertical bars, ($n = 8$). Mean values indicated by single asterisk were significantly different compared with the control group ($p < 0.05$). Repeated-measures, 2-factor ANOVA was used to analyze H_2 excretion between the control and 20% HAS groups across time: (A) HAS, $p < 0.0001$; time, $p = 0.0001$; interaction, $p = 0.0009$; (B) HAS, $p < 0.0001$; time, $p = 0.3850$; interaction, $p = 0.3710$. 20% HAS, rats fed the control diet supplemented with 20% high amylose cornstarch; ZT, Zeitgeber time.

maintain high colonic H_2 production. Some investigators reported H_2 production by colonic fermentation of non-digestible saccharides *in vitro*[18]. However, *in vitro* fermentation would be different from *in vivo* fermentation because fermentation substrates are not continuously supplied and the fermentation products are eliminated by absorption. Therefore, we examined changes in colonic H_2 production over a 24-h period in rats fed HAS.

Materials and methods

Samples. HAS (Hi-maize 1043; total dietary fiber, 64.5%,[19] resistant starch, 45.7%[19], and amylose, ~70%) was kindly supplied from Nippon NSC Ltd. (Tokyo, Japan).

Animals and diets. This study was approved by the Nayoro City University Animal Use Committee (No.11-04), and animals were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals, Nayoro City University. Seven-week-old, male Sprague-Dawley rats weighing 210–230 g were obtained from Japan SLC (Haruno colony; Shizuoka, Japan). They were housed in individual cages with screen bottoms made of stainless steel in a room maintained at 23 ± 1 °C with humidity ranging from 50% to 70% under lighting conditions with 12 h of light (0700–1900) and 12 h of darkness daily. Rats were acclimated by feeding a 25% casein-control (C) diet[20] (Supplemental Table 1) for 7 or 10 days in all experiments.

In vivo *digestibility of HAS and colonic utilization of the resistant starch fraction in HAS.* To determine the availability of HAS in the small and large intestines, we examined the *in vivo* digestibility of HAS in the small intestine and colonic utilization of the resistant starch fraction in HAS in ileorectostomized rats and sham-operated rats. Rats were deprived of food for 24 h before surgery although they had access to water. After the acclimation period, 25 rats were subjected to ileorectostomy (n 13) or sham-operation (n 12) under anesthetized conditions as described in our previous report.[21] The operation was performed according to the method of Lambert[22] with modifications.[23] As described by Morita et al.,[23] to shorten the recovery period, we did not dissect the cecum and the colon, but the ileocecal valve was ligatured (closed), and then the colonic terminal was anastomosed to the stoma in the abdominal wall to allow the cecal and colonic contents to be excreted naturally. The rats were intraperitoneally administered sulfamethoxazol (5 mg; Shinomin, Shionogi, Tokyo) in order to prevent infections during the surgery. They were not allowed food for the first 24 h postoperatively, and then were fed the control diet for 14 days although the growth rates in operated rats became the same as those in the sham-operated rats only after 3 d. Following the postsurgical recovery period, ileorectostomized rats and sham-operated rats were assigned to 2 subgroups ($n = 7$ or 6) based on body weight. One set of ileorectostomized and sham-operated groups was fed the control diet or the 20% HAS diet for 7 d. For determination of the starch content excreted into feces, feces were collected during the last 3 d of the experimental period, lyophilized, weighed, and stored at −30 °C.

Calculations. Starch digestibility from mouth to anus was calculated using the data of sham-operated rats fed the control diet by the following formula

$$\text{Starch digestibility}_{\text{mouth}-\text{anus}} = (\text{starch intake} - \text{fecalstarch}/\text{starch intake}) \times 100$$

The starch digestibility from mouth to small intestine was calculated by the above formula using the data of ileorectostomized rats fed the control diet.

HAS digestibility was calculated by the above formula using HAS intake and fecal starch content of rats fed the 20% HAS diet. Fecal starch content derived

from HAS was calculated by subtracting that in rats fed the control diet from that in rats fed the 20% HAS diet.

Starch utilization in the large intestine was calculated by the following formula:

$$\text{Starch utilization in the large intestine} = (\text{starch intake} - \text{digestible starch in the small intestine} - \text{fecal starch})/\text{starch intake}) \times 100$$

HAS utilization in the large intestine was calculated by replacing starch parameters with parameters of starch derived from HAS.

Experiment 1. To determine the daily fluctuation of colonic H_2 production upon ingesting HAS, we examined the time course of the change in (breath + flatus) H_2 excretion during a 24-h period in rats fed the diet containing HAS. After the acclimation period, 18 rats were assigned into 2 groups ($n = 9$) based on body weight and (breath + flatus) H_2 excretion, and were given a diet supplemented with or without 200 g of HAS per kg for 28 days. Supplementation of HAS was done by replacing an equal weight of cornstarch in the C diet. (Breath + flatus) H_2 excretion was measured every 4 h during a 24-h period prior to d 0, and on d 7, 14, and 28 in rats, which were fed the respective diet only during the dark period. Measurement was started at Zeitgeber time 0 (ZT 0), which was defined as the onset of the light period, on the respective day.

Experiment 2. To clarify whether colonic H_2 production can be maintained for 24 h with an insufficient supply of fermentation substrate, we examined whether H_2 excretion is maintained for 24 h when the amount of resistant starch in ingested HAS is equal to the amount of resistant starch utilized in the large intestine of rat over 24 h. After the acclimation period, 15 rats were assigned into 2 groups ($n = 7$ or 8) based on body weight and (breath + flatus) H_2 excretion, and were given a diet supplemented with or without 100 g of HAS per kg for 14 days. Supplementation of HAS was done by replacing an equal weight of cornstarch in the C diet. (Breath + flatus) H_2 excretion was measured every 4 h during a 24-h period on d 14 in rats that had been fed the respective diet only during the dark period. Measurement was started at ZT 0, which was defined as the onset of the light period, on the respective day.

Sampling. (Breath + flatus) H_2 excretion per 5 min was measured by placing the rat inside a sealed polypropylene chamber for 5 min. After 5 min, an appropriate volume of the gaseous phase was withdrawn using a gas-tight syringe for analysis of H_2 in breath and flatus. At the end of the experiment, rats were anesthetized with sodium pentobarbital (50 mg/kg body weight). After immediate laparotomy, 1 ml of blood was removed from the portal vein and sealed in heparin vials for H_2 analysis. The H_2 concentration was determined using a GC analyzer (lower detection limit, 0.10 ppm; quantification range, 0.30–50 ppm; Biogas analyzer BAS-1000; Mitleben, Osaka, Japan).

Analyses. The H_2 concentration in the gaseous phase was immediately determined using the GC analyzer (Biogas analyzer BAS-1000; Mitleben) after sampling. Fecal starch content was measured using a commercial kit (Total Starch Assay Kit; Megazyme Inc., Sydney, Australia) according to the manufacturer's protocol.

Statistical analysis. Values obtained from the experiments were expressed as means with their standard errors or medians with range. Data were subjected to Bartlett's test for homogeneity of variances, and unequal variances were stabilized by log transformation. For samples with equal variances, either one-way or two-way ANOVA was used, followed by the Tukey–Kramer *post hoc* test for multiple comparisons between individual group means. If sample variances were still unequal after log transformation, we used the Wilcoxon test and the Steel–Dwass test. Repeated-measures 2-way ANOVA was used to analyze the change in H_2 excretion over time between the control and HAS groups. H_2 excretion within an individual group was compared with repeated-measures 1-way ANOVA. The Tukey–Kramer test, Steel–Dwass test, and Wilcoxon test were performed using SAS JMP software (version 9.0.2; Tokyo, Japan). Significance was defined as $p < 0.05$.

Results

In vitro digestibility of HAS and colonic utilization of the resistant starch fraction in HAS

Body weight gain during the experimental period was significantly lower in ileorectostomized rats fed the 20% HAS diet than in those fed the control diet although food intake did not differ among the 4 groups. However, there were no differences in growth rate on the last 3 days among the 4 groups: 15 ± 1 g/3 d in the sham-C group, 16 ± 2 g/3 d in the ope-C group, 16 ± 1 g/3 d in the sham-HAS group and 14 ± 2 g/3 d in the ope-HAS group. Cornstarch was almost completely digested in the small intestine and few resistant fractions were present (Table 1). Calculating the digestibility of HAS in the small intestine, the digestibility of HAS in the small intestine was 53.0% in rats fed the 20% HAS diet, and 47.0% of the ingested HAS was supplied into the large intestine. 24.3% of the ingested HAS (51.8% of the supplied resistant fraction), which corresponded to 1.04 g/day, was utilized in the large intestine.

Colonic H_2 production in rats fed the 20% HAS diet (Experiment 1)

We examined the time course of the change in (breath + flatus) H_2 excretion during a 24-h period in rats fed the diet with or without 200 g of HAS per kg for 28 days. Food intake and body weight gain did not

Table 1. Food intake, body weight gain, fecal parameters, and starch digestibility in sham-operated and ileorectostomized rats fed a diet with or without 20% HAS.

	Sham-C	Ope-C	Sham-HAS	Ope-HAS
Food intake (g/7 days)	165 ± 6	158 ± 2	160 ± 7	155 ± 5
Body weight gain (g/7 days)	34 ± 2^{ab}	40 ± 4^{a}	38 ± 3^{ab}	27 ± 3^{b}
Starch intake[1] (g/3 days)				
Total	33.8 ± 1.1	30.9 ± 1.5	32.4 ± 1.4	31.1 ± 1.4
Derived from HAS	–	–	13.4 ± 0.6	12.9 ± 0.6
Feces dry weight (g/3 days)	6.31^{c} (6.00–7.11)	7.35^{bc} (6.41–7.96)	9.42^{b} (7.00–11.5)	13.0^{a} (10.6–14.9)
Fecal starch (g/3 days)	0.031^{d} (0.027–0.034)	0.144^{c} (0.116–0.160)	3.21^{b} (1.00–5.43)	6.14^{a} (4.74–7.16)

	Control diet	20% HAS diet
Starch digestibility[2] (%)		
Total starch		
Mouth−small intestine[3]	99.5 ± 0.0	80.1 ± 1.5***
Mouth−anus[3]	99.9 ± 0.0	90.5 ± 1.9***
Colonic utilization[3]	0.4 ± 0.0	10.5 ± 1.5***
Starch derived from HAS		
Mouth−small intestine	–	53.0 ± 5.3
Mouth−anus	–	77.4 ± 4.5
Colonic utilization	–	24.3 ± 3.5

Notes: Data are expressed as the means ± SE or medians (minimum values-maximums), $n = 7$ or 6. Mean and median values within a row with unlike superscript letters were significantly different ($p < 0.05$). Sham-C and Ope-C, sham-operated and ileorectostomized rats fed the control diet, respectively; Sham-HAS and Ope-HAS, sham-operated and ileorectostomized rats fed the control diet supplemented with 20% high amylose cornstarch.
Two-way ANOVA results were as follows: Ope, $p = 0.2762$; HAS, $p = 0.4610$; interaction, $p = 0.8163$ for food intake, Ope, $p = 0.3697$; HAS, $p = 0.1476$; interaction, $p = 0.0079$ for body weight gain and Ope, $p = 0.1388$; HAS, $p = 0.6460$; interaction $p = 0.5748$ for total starch intake.
[1]Total starch and HAS intake were calculated using food intake, the added amount of starch and HAS to the diet, respectively.
[2]Starch digestibility was calculated using starch intake and fecal starch excretion in sham-operated and ileorectostomized rats fed the control diet or 20% HAS diet for 3 days.
[3]Mean values indicated by triple asterisk were significantly different compared with that in the control group ($p < 0.001$).

Table 2. Intake of food, HAS and resistant starch, body weight gain, H_2 excretion in breath and flatus, and portal H_2 concentration in rats fed the 20% HAS diet.

	Control	20% HAS
Food intake (g/28 d)	563 ± 23	536 ± 23
HAS intake[1] (g/28 d)	–	107 ± 5
RS intake[1] (g/28 d)	–	51.5 ± 2.2
Body weight gain (g/28 d)	123 ± 8	118 ± 9
24-h H_2 excretion in breath and flatus[2](μmol)		
d 0	34.9 (16.4–51.4)	28.6 (11.7–59.6)
d 7	20.2 (10.5–55.9)	286* (61.6–999)
d 14	33.1 (12.6–79.8)	378** (45.6–1430)
d 28	25.5 (9.1–80.4)	469* (126–1900)
Portal H_2[2] (μmol/L)	0.117 (0.011–1.48)	2.21** (0.256–4.51)

Notes: Data are expressed as the means ± SE or medians (minimum values-maximums), $n = 9$. Median values indicated by single or double asterisk were significantly different compared with that in the control group ($p < 0.05$ or 0.01, respectively). 20% HAS, group fed the control diet supplemented with 20% high amylose cornstarch; HAS, high amylose cornstarch; RS, resistant starch.
[1]HAS intake and RS intake were calculated using food intake, the added amount of HAS to the diet and starch digestibility as shown in Table 1.
[2]Mean values indicated by single and double asterisk were significantly different compared with that in the control group ($p < 0.05$ and $p < 0.001$, respectively).

differ between the two groups (Table 2). However, food intake on the day of analysis for 24-h H_2 excretion in breath and flatus was 70−80% of those on the day before and after analysis. Intake of resistant fraction in HAS during last 24 h of the experiment was 1.57 ± 0.10 g in the 20% HAS group. From d 7, H_2 excretion in breath and flatus during a 24-h period in the 20% HAS group showed higher values than in the control group although the difference did not reach statistical significance at several time points (Fig. 1(A)–(D)). In the 20% HAS group, 24-h H_2 excretion in breath and flatus was 19 times higher on d 14 compared with that on d 0, although H_2 excretion in the control group, which were fed cornstarch and sucrose as carbohydrate sources, was maintained at about 30 μmol throughout the experimental period (Table 2). H_2 excretion at ZT 24 on d 28 was exponentially correlated with portal H_2 concentration (Fig. 2(A)). Portal H_2 concentration was estimated to be higher in rats fed the 20% HAS diet for 24 h although the concentration would be at a minimum level at ZT 16 (Fig. 2(B)).

Colonic H_2 production in rats fed the 10% HAS diet (Experiment 2)

We examined whether H_2 excretion is maintained for 24 h when the amount of resistant starch in ingested HAS is equal to the amount of resistant starch utilized in the rat large intestine over 24 h. Food intake and body weight gain did not differ between the two groups (Table 3). As in experiment 1, food intake in the control and 10% HAS groups on the day of analysis for 24-h H_2 excretion in breath and flatus was 81.1 ± 3.4% and 76.6 ± 1.8% of those on the day before analysis,

Table 3 Intake of food, HAS and resistant starch, body weight gain, H_2 excretion in breath and flatus, and portal H2 concentration in rats fed the 10% HAS diet[a].

	Control	10% HAS
Food intake (g/14 d)	312 ± 5	332 ± 10
HAS intake[1] (g/14 d)	–	33.2 ± 1.0
RS intake[1] (g/14 d)	–	15.6 ± 0.5
Body weight gain (g/14 d)	71.5 ± 2.9	79.9 ± 5.2
24-h H_2 excretion[2] (μmol)	58.1 (32.0–95.7)	278** (146–1100)
Light period[2]	31.0 (11.6–48.1)	147** (36.8–912)
Dark period[2]	38.3 (20.4–67.5)	145** (52.8–214)
H_2 excretion $AUC_{d\ 0–14}$[2] (mmol)	0.898 (0.505–3.68)	8.93** (5.52–12.4)
Portal H_2[2] (μmol/L)	0.122 (0.055–0.583)	0.969** (0.248–5.66)

Notes: Data are expressed as the means ± SE or medians (minimum values-maximums), $n = 8$. Mean values indicated by double asterisk were significantly different compared with that in the control group ($p < 0.01$). 10% HAS, group fed the control diet supplemented with 10% high amylose cornstarch; HAS, high amylose cornstarch; RS, resistant starch.

[1]HAS intake and RS intake were calculated using food intake, the added amount of HAS to the diet and starch digestibility as shown in Table 1.

[2]Mean values indicated by double asterisk were significantly different compared with that in the control group ($p < 0.001$).

respectively. Intake of resistant fraction in HAS during last 24 h of the experiment was 0.89 ± 0.03 g in the 10% HAS group. From d 3, H_2 excretion in breath and flatus and portal H_2 concentration were significantly higher in the 10% HAS group than in the control group (Table 3, Fig. 3(A)). Unlike in the 20% HAS group (experiment 1), on d 14, the H_2 excretion in the 10% HAS group was not maintained at a constant level throughout a 24-h period, although the H_2 excretion was higher than that in the control group except at ZT 4 (Fig. 3(B)). The H_2 excretions in the 10% HAS group at ZT 12 and 16 were less than 0.9 μmol/5 min.

Discussion

In the present study, in rats fed the 20% HAS diet, 53.0% of the ingested HAS was digested in the small intestine and then 24.3% of that was utilized by colonic bacteria. From these data and HAS intake during last 3 d of the experiment period, it is suggested that 2.02 g of the resistant fraction in HAS was delivered to the large intestine, 1.04 g of which was utilized by colonic bacteria over a 24-h period. Therefore, if a sufficient amount of the resistant fraction is not supplied, less colonic H_2 would be produced because of the lack of a fermentation substrate. Considering the digestibility of HAS in the small intestine, 1.09 g of the resistant fraction, which corresponds to the amount utilized by colonic bacteria for 24 h, could be delivered into the large intestine by feeding the 10% HAS diet. Actually, in experiment 2, 0.89 g of the resistant fraction was administrated by feeding 10% HAS diet because of lowered food intake due to analysis for 24-h H_2 excretion. H_2 excretion in the group fed the 10% HAS diet gradually decreased after ZT 4 during the light period and then showed the lowest value at ZT 12, while the excretion gradually increased as time elapsed during the dark period. This supports the above assumption.

In the present study, compared with the control group, higher H_2 excretion in breath and flatus was maintained for 24 h in the group fed the 20% HAS diet on d 7, 14, and 28. These results indicate that administration of HAS by feeding the 20% HAS diet can be sufficient to induce the production of a high level of colonic H_2 for 24 h. Total H_2 excretion in the 20% HAS group on d 28 was calculated as 469 (range 126–1900) μmol/24 h from the $AUC_{ZT\ 0–24}$. The amounts of HAS, resistant starch, and hexose unit that were supplied to the large intestine could be estimated from food intake on d 28. Using these estimations, H_2 excretion was calculated as 117 (range 28.8–547) μmol per gram of HAS intake, 480 (118–2250) μmol (10.8 (range 2.6–50.4) mL) per gram of resistant starch, and 80.7 (range19.9–378) mmol per mol of hexose. Moreover, it was estimated that only 1.35 (range 0.33–6.31)% of the hydrogen atoms in resistant starch utilized in the large intestine was excreted as hydrogen gas. These data correspond to those in a human study. H_2 excretion was 7.9–17.3 mL/g of lactulose in human subjects.[24] The amount of H_2 production in the large intestine should be more than the amount of H_2 excreted because all of the H_2 supplied into the body is not excreted. H_2 is utilized for scavenging of reactive oxygen species[25] and as a fermentation substrate of methanogen, sulfate-reducing bacteria and homoacetogen.[26] Also, in the present study, food intake on the date of measurement of 24-h H_2 excretion was about 70% of that on other days because breath and flatus H_2 excretion was measured every 4 h during a 24-h period. H_2 excretion in rats fed the 20% HAS diet was about 70% higher in our previous study[7] than in the present study. Therefore, H_2 excretion in the present study would be underestimated due to the unavoidable reduced food intake.

H_2 molecules can be delivered to the body by inhaling H_2 gas, administering H_2 water and producing colonic H_2. Based on the rat tidal volume (1 mL) and respiratory rate (85 times/min), inhalation of 4% H_2 gas would introduce 220 mmol of H_2 per day to the lungs. Ohsawa et al. reported that the arterial H_2 concentration in rats reached 18 μmol/L (H_2 partial pressure, 17 mm Hg) by inhaling 4% H_2 gas (H_2 partial pressure in alveolar air, 29 mm Hg).[6] Therefore, 60% of the inhaled H_2 molecules, that is, 132 mmol of H_2 would enter into the blood by gas exchange in the lungs. Although this is a large amount, it is difficult to administer H_2 in daily life by this means. On the other hand, when administering H_2 water, we estimated the amount of supplied H_2 to be 28 μmol per day based on the amount of drinking in rats (35 mL/day) and the saturated H_2 concentration in water (800 μmol/L).

This low supply of H_2 could not continuously maintain a high level of H_2 in the body. According to the results on d 28 in the present study, the amount of colonic H_2 derived from HAS was 469–670 (70% higher value) μmol per day. Although the supply of H_2 by colonic H_2 is quantitatively inferior to that by inhalation of H_2, the former means can continue to deliver H_2 molecules in the body if colonic fermentation is activated by non-digestible saccharides such as HAS. Therefore, colonic H_2 has an advantage over other means regarding the continuous delivery of H_2, which has an antioxidative effect, in the body. The amount of colonic H_2 produced varies depending on the type of non-digestible saccharides ingested; therefore, further studies are required to evaluate the relationship between colonic H_2 production and different non-digestible saccharides.

H_2 molecules in portal blood travel through the bloodstream to the heart and then to the lungs. A considerable amount of H_2 is excreted from the lungs into breath by gas exchange. Therefore, there should be a correlation between portal H_2 concentration and breath H_2 excretion. In the present study, portal H_2 concentration was significantly logarithmically correlated with H_2 excretion in breath and flatus (Fig. 2(A)). Using the formula and H_2 excretion, the time-course of portal H_2 concentration over a 24-h period is estimated as shown in Fig. 2(B). Then, the total amount of H_2 passing through the portal vein over 24 h (0.0 (range 0.0–11.8) μmol/24 h vs. 50.9 (range 21.1–104) μmol/24 h in the control and 20% HAS groups, respectively) could be calculated from the AUC of the time course and portal flow velocity (20.0 mL/min) that had been previously measured in rats with the same body weight using a Laser Flowmeter (ALF21D, Advance Co., Tokyo, Japan). These results showed that about 11% of the excreted H_2 would be via the lungs in breath in rats fed the 20% HAS diet and the rest would be from flatus. The proportion of H_2 that is exhaled in breath has been reported to be 14–88% in humans,[24,27] but has not been reported in rats. The proportion of H_2 excreted in breath is low when much more H_2 is produced.[24] Therefore, colonic H_2 in rats fed HAS might be predominantly excreted as flatus because the H_2 production was enhanced by administration of HAS, which is relatively rapidly fermented in the large intestine.[28,29]

Net production of colonic H_2 is dependent on the balance between production and utilization of H_2 by microbes.[30] H_2-producing bacteria include *Ruminococcus* spp., *Roseburia* spp., *Clostridium* spp., and *Bacteroides* spp., while H_2-utilizing bacteria include methanogens, reductive acetogens, and sulfate-reducing bacteria.[30] The microbiota of laboratory animals differ among breeders and breeding colonies.[31] As described in our previous study, H_2 excretion in breath and flatus varied even with the administration of the same fermentation substrate to rats from the same breeder. The difference in H_2 excretion is assumed to reflect the balance between H_2-producing bacteria and H_2-utilizing bacteria. In the present study, colonic microbiota was not examined, therefore further investigation is required to evaluate the relationship between microbiota, various non-digestible saccharides and colonic H_2 production. The results of these studies could contribute to stable H_2 production by diverse human microbiota. Lower limit of H_2 to alleviate oxidative stress should be determined because excess production of colonic H_2 could leads to the side effects such as bloating, belching, and flatulence.[32] The results in the present study should give useful information to determination of requirements of non-digestible saccharides for appropriate colonic H_2 production.

We found in the present study that high production of colonic H_2 was maintained over a 24-h period when sufficient amounts of HAS were delivered to the large intestine as a fermentation substrate. This finding would represent the effectiveness of H_2 delivery into the body due to colonic H_2 derived from non-digestible saccharides such as HAS. Continuous delivery of H_2 will provide a significant means for antioxidant therapy and prevention.

Author contributions

NN designed the study; NN, HT, and TY conducted the study; NN analyzed the data-set; NN wrote the manuscript; and NN had primary responsibility for the final content. All authors were involved in designing the study, reviewing and interpreting the results, and drafting the manuscript. All authors read and approved the final manuscript.

Disclosure statement

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Supplemental materials

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