Quantitative Assessment of Effective Energy of Resistant Cornstarch Using $^{13}CO_2$, H_2, and CH_4 Breath Tests

Maki Kinoyama, Hayami Nitta, Yuko Hatase, and Akiharu Watanabe*

Department of Clinical Nutrition, Faculty of Health Science and Technology, Kawasaki University of Medical Welfare, 288 Matsushima, Kurashiki, Okayama 701–0193, Japan

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We calculated the effective energy of a resistant cornstarch (HB-450) by using $^{13}CO_2$, H_2, and CH_4 breath tests to assess HB-450 digestion and fermentation in healthy female subjects. On the basis of the areas under the curves (AUCs) for the concentrations of $^{13}CO_2$ and H_2 following ingestion of 40 g of HB-450, 23.0 ± 6.8 g was digested in the upper digestive tract and 3.6 ± 2.1 g was fermented by intestinal flora. The effective energy of the 40 g dose was thus calculated as 2.5 ± 0.8 kcal/g. These results indicate that $^{13}CO_2$, H_2, and CH_4 breath tests are useful for measuring the effective energy of resistant cornstarch.

Key words —— resistant cornstarch, digestion, effective energy, fermentation, respiratory gas analysis ($^{13}CO_2$, H_2, CH_4)

INTRODUCTION

Starch is an important source of energy for human metabolism. As such, it is essential to calculate the effective energy of starch in the diets of patients with metabolism disorders such as diabetes mellitus. According to early calculations of the Atwater factor,[1] 1 g of ingested starch provides 4 kcal of effective energy. However, it was found during the 1980s that not all starch is broken down by pancreatic amylase in the human digestive tract.[2] Starch that is not broken down by this enzyme has become known as resistant starch (RS).[3] RS is the generic name for starch and its metabolites that are not digested in the small intestine of a healthy person. The identification of RS resulted in a major change in the definition of dietary fiber. RS is now classified nondigestible starch and referred to, when present in the lumen of the human digestive tract, as a luminacoid.[4]

Some types of RS are fermented by enteric bacteria in the colon, leading to the production of gases such as H_2, CH_4, and CO_2 and of organic acids including lactic acid and several short-chain fatty acids such as acetic acid, butyric acid, and propionic acid. These acids are partially absorbed from the colon and supply energy to large intestinal mucosal cells. However, some types of RS are excreted unchanged in the feces in the manner of insoluble dietary fiber. It thus appears that different types of RS differ in the degree to which they are digested or fermented,[5] although it has proved difficult to quantify *in vivo* the extent to which an orally ingested starch is digested or its residue fermented by enteric bacteria and to identify what gases and organic acids are produced during these processes.[6–8] As a result, it has not to date been possible to calculate the amount of effective energy provided by luminacoids such as RS.[9]

One method that has been reported reliable for evaluating *in vivo* the quantity of fermentation of RS involves the simultaneous measurement of the concentrations of H_2 and CH_4 in exhaled breath.[10, 11] Another method is based on the fact that C4 plants such as corn have carbon-fixing enzymes that can absorb, during photosynthesis, large quantities of the naturally occurring and stable isotope ^{13}C. By measuring the amounts of $^{13}CO_2$ and $^{12}CO_2$ in exhaled breath and calculating the $^{13}CO_2/^{12}CO_2$ ratio, it is possible to obtain quantitative estimates of the amounts of a RS that are digested.[12] These two methods can be simultaneously used for measuring the effective energy of RS that is digested and fermented in the human digestive tract.

In the present study, we calculated the effective energy of a RS, specifically, resistant cornstarch (HB-450), by simultaneously measuring the concentrations of $^{13}CO_2$, H_2, and CH_4 in the exhaled air of healthy human subjects. This calculation is essential for nutritional assessment in patients with lifestyle-related diseases.

*To whom correspondence should be addressed: Department of Clinical Nutrition, Faculty of Health Science and Technology, Kawasaki University of Medical Welfare, 288 Matsushima, Kurashiki, Okayama 701–0193, Japan. Tel: +81-86-462-1111 (ext. 54052); Fax: +81-86-463-3508; E-mail: awat@mw.kawasaki-m.ac.jp

MATERIALS AND METHODS

Subjects —— The subjects were three female students (average age: 22.8 years, and BMI: 21.2), who were selected from among 8 students by confirming a good reproducibility (serial course of $^{13}CO_2$, H_2 and CH_4 concentrations in the breath) of digestibility and fermentability of RS.[13] This is due to the fact that the enterobacterial flora differs greatly among subjects. Therefore, in this study, the three subjects whose dietary intakes and bacterial flora in the gut are stably constant were tested in preliminary experiments. They also showed only slight symptoms even after 40 g of lactulose was orally ingested and none of them was a CH_4 producer. Only those subjects who agreed to take part in the study on the basis of a full understanding of its background, aims, and methods of the study and its foreseeable detrimental physical effects were enrolled in the study.

The study conformed to the Declaration of Helsinki regarding use of human subjects and was approved by the Ethics Committee of Kawasaki University of Medical Welfare (Approval Number 021).

Materials —— The test material (AmyloGel HB-450, Sanwa Starch Co., Ltd., Nara, Japan, abbreviated as HB-450) used in the study consisted of high amylose cornstarch, which was heat-moisture-treated to yield a highly stable HB-450. The content of dietary fiber in this material was estimated as 64.5% by the Prosky method (*in vitro*).[14,15] This cornstarch is classified as RS3 which is referred to as retrograded starch.[16,17] Other materials used in the study were amylopectin, an easily digestible glutinous, waxy cornstarch (Sanwa Starch Co., Ltd.) which was the control starch used in this study, and a nondigestible disaccharide, lactulose (Nutrition Science Laboratories of Morinaga Milk Co., Ltd., Zama, Japan).

Study Design —— Each subject underwent carbohydrate load testing starting early in the morning after a fast that began at 9.00 p.m. the previous day. Subjects were told to avoid food products containing cornstarch and those with high levels of nondigestible oligosaccharides and dietary fiber during the day before the test.

On the morning of the study, baseline breath tests were obtained by sampling each subject's breath three times at 15-min intervals in a breath bag. After stable baseline measurements of $^{13}CO_2$, H_2, and CH_4 concentrations had been obtained, the subject was given 40 g of one of the carbohydrates mixed in 100 ml of water to drink. Every 30 min for the next 15 hr the subject's breath was collected in the breath bag. Subjects consumed 200 g of white boiled rice to prevent hunger 5.5 hr and 11.0 hr after the oral carbohydrate load, and were permitted to drink tap water *ad libitum*. The boiled rice was previously confirmed not to affect the concentration of H_2, CH_4 or $^{13}CO_2$ in the breath during the test. Each subject underwent 3 days of testing: 1 day for HB-450, 1 day for waxy starch, and 1 day for lactulose. Test days were separated by 2 days or more.

Breath $^{13}CO_2$ Analysis (Digestibility Test) —— To obtain the sample for testing $^{13}CO_2$ concentration, the subject was told to hold the breath for about 15 sec and then to exhale; the final approximately 300 ml of air exhaled (end tidal air) was collected via a mouthpiece placed inside the mouth with the other end in the breath bag.

The proportion of $^{13}CO_2$ ($^{13}CO_2/^{12}CO_2$) in the breath was analyzed by infrared spectroscopy using a POCone breath $^{13}CO_2$ analyzer (Otsuka Pharmaceutical Co., Ltd., Osaka, Japan). The proportion of $^{13}CO_2$ was expressed as parts per thousand (‰) using Pee Dee Belemnite (PDB) international standards for calcium carbonate.

Breath H_2 and CH_4 Analysis (Fermentation Test) —— The method used to collect breaths for analyzing H_2 and CH_4 concentrations was a 2-bag method. The subject was instructed to breathe lightly, then to hold the breath for about 15 sec, and then to exhale into a mouthpiece placed inside the mouth with the other end in the tip of a breath bag. After about 450 ml of breath equivalent to dead space had been exhaled into the first bag, the stopcock to the second bag was opened and about 100 ml of end tidal air was collected in the second bag. From the second bag, 20 ml of air was withdrawn into a syringe to be used with the breath gas sampling system.

Breath samples were analyzed by gas chromatography using the Model TGA 2000 breath gas analyzer (Teramecs Co., Ltd., Kyoto, Japan). In most cases, breath samples were analyzed the same day, and in every case analysis was completed no later than 2 days after the breath had been collected.

For sample analysis, the contents of the syringe were injected into the breath gas analyzer and the concentrations of H_2 and CH_4 were measured simultaneously over the course of 5 min. The Model TGA 2000 breath gas analyzer has a lower limit for detection of each of the two gases of 1 ppm with

reproducibility of ±2 ppm and linear calculation of results over time for concentrations of either gas of up to 100 ppm. A test mixture gas containing both 100 ppm of H_2 and 50 ppm of CH_4 was used once per hr to calibrate the breath gas analyzer while samples were being analyzed.[18)]

Simultaneous analysis of the concentrations of H_2 and CH_4 is based on the principle that methane is produced mainly from hydrogen by methanogens such as *Methanobrevi-bacter smithii*.[13)]

Calculations of Starch Digestibility, Fermentation, and Effective Energy —— The values obtained for proportion of exhaled $^{13}CO_2$, H_2, and CH_4 at each measurement time were entered into a computer software spreadsheet program for calculating the effective energy of HB-450 using the following equations (Excel 2003, Microsoft Corp., Redmond, WA, U.S.A.).

The area under the curve (AUC) was calculated for the proportion of exhaled $^{13}CO_2$ and for exhaled H_2, CH_4 over time using the trapezoid method.[19)] The AUCs were used to calculate digestibility, fermentation, and effective energy of HB-450.

For the following equations, the reference/control values were as follows:

- for complete digestibility (100%): the AUC for exhaled $^{13}CO_2$ after ingestion of 40 g of waxy starch, using 4 kcal/g as the energy supplied; and
- for complete fermentation (100%): the AUC of exhaled H_2 after ingestion of 40 g of the nondigestible disaccharide lactulose; 2 kcal/g was used as the energy supplied by the organic acids that appear in fermentation of 1 g lactulose.[9)]

The digestibility of HB-450 was calculated by measuring the proportion of $^{13}CO_2$ in the breath using waxy starch as the control of complete digestibility (100%).

$$\text{The digestibility of HB-450 (\%)} = \left\{\frac{\text{AUC of HB-450 (‰·min)}}{\text{AUC of waxy starch (‰·min)}}\right\} \times 100(\%) \quad (1)$$

The fermentation of HB-450 was calculated by measuring the proportions of H_2 and CH_4 in the breath using lactulose as the control of complete fermentation (100%).

$$\text{The fermentation of HB-450(\%)} = \left\{\frac{\text{AUC of HB-450 (ppm·min)}}{\text{AUC of lactulose (ppm·min)}}\right\} \times 100(\%) \quad (2)$$

From eqs. (1) and (2), the effective energy of HB-450 was calculated according to the following equation:

$$\begin{aligned}\text{Quantitative effective energy of HB-450} &= \{\text{HB-450 digestivility (\%)} \times 4\,\text{(kcal/g)}\} \\ &\quad + \{\text{HB-450 fermentation (\%)} \times 2\,\text{(kcal/g)}\}\end{aligned} \quad (3)$$

Statistical Analyses —— Results were expressed as mean and standard deviation (S.D.). Statistical differences were calculated using 2-sided Student's *t*-test by Microsoft Excel®. Differences were considered significant at $p < 0.05$ between the results of AUC for HB-450 *vs*. lactulose or HB-450 *vs*. waxy starch.

RESULTS

The peak of $^{13}CO_2$ in the breath was 2.7 ± 0.7‰ at 3.8 ± 1.3 hr after ingestion of 40 g of HB-450 (Fig. 1). The AUC for the proportion of $^{13}CO_2$ after HB-450 ingestion was 57.5 ± 16.9% of the AUC for this ratio after ingestion of waxy starch. The ratios in eq. (1) indicate the digestibility of HB-450 (%).

The H_2 concentration in exhaled breath began to increase 5 hr after ingestion of 40 g of starch and the increase was maintained for up to 15 hr after ingestion (Fig. 2). The increase after ingestion of HB-450 or waxy starch was minimal, but after ingestion of lactulose, the H_2 concentration peaked at almost 300 ppm. H_2 concentration after ingestion of HB-450 peaked at 35.7 ± 0.6 ppm. The AUC after ingestion of 40 g of lactulose was 10573 ± 3844. The AUC after ingestion of HB-450 was 8.9 ± 7.2% of the AUC for lactulose. The ratios in eq. (2) indicate the fermentation of HB-450 (%).

According to eq. (3), of the 40 g of HB-450 ingested by subjects in this study, the digestibility was 57.5 ± 16.9% and the fermentability was 8.9 ± 7.2%, and thus the effective energy of the 40 g dose of HB-450 was 2.5 ± 0.8 kcal/g.

DISCUSSION

In the *in vivo* study reported here, the effective energy of HB-450 was calculated to be 2.5 kcal/g, which is comparable with 2.7 kcal/g calculated by the Prosky method (*in vitro*).[20)] To obtain this theoretical value of 2.7 kcal/g, we calculated the effec-

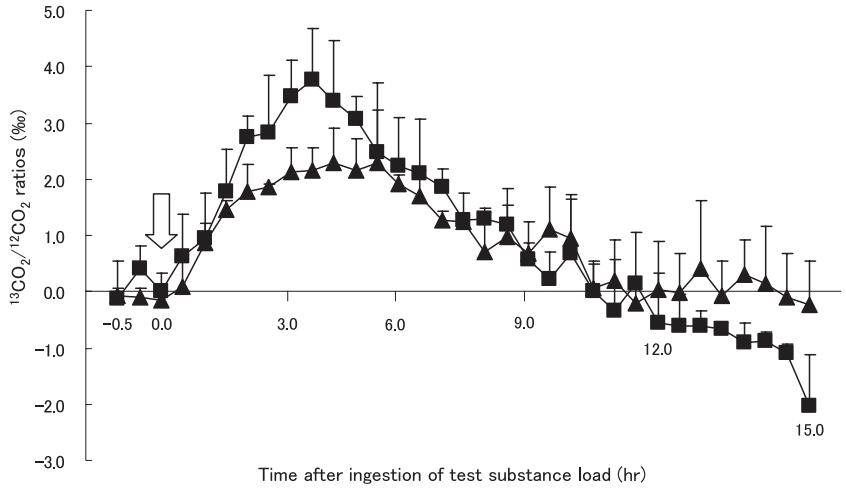

Fig. 1. Proportion of $^{13}CO_2$ ($^{13}CO_2/^{12}CO_2$) in Exhaled Breath Measured Every 30 Minutes after Ingestion of 40 g of HB-450 or 40 g of Waxy Starch (Control)

The arrow shows the time of test substance ingestion. Each data point represents the mean and each vertical bar represents 1 S.D. above the mean for 3 subjects. ▲: HB-450, ■: waxy starch.

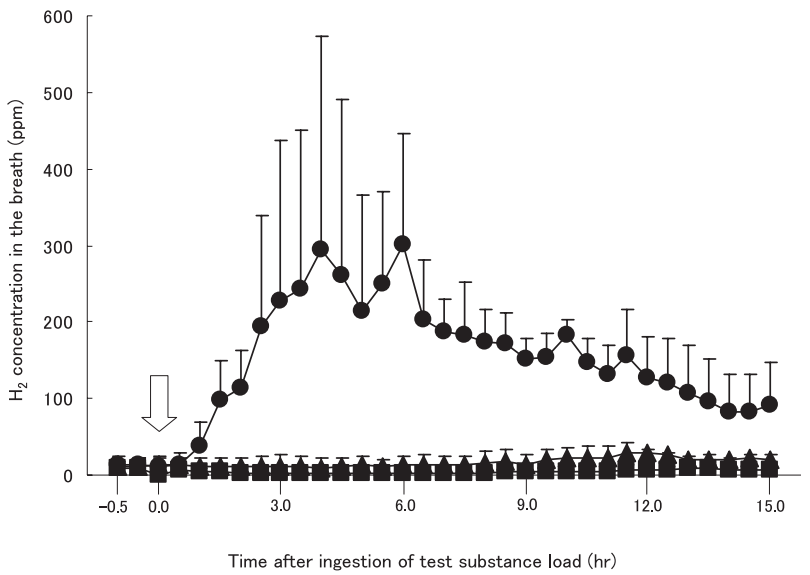

Fig. 2. Concentrations of H_2 and CH_4 in ppm in Exhaled Breath Measured Every 30 Minutes after Ingestion of 40 g of HB-450 or 40 g of Waxy Starch or 40 g of Lactulose (Control)

Because the three subjects were not methane producers, breath CH_4 concentration was simultaneously measured in all samples. However, because every sample contained about 1 ppm of CH_4, very similar to the concentration inside the laboratory, CH_4 concentration is not shown in this figure. Lactulose was used as the control of complete fermentability (100%). The arrow shows the time of test substance ingestion. ▲: HB-450, ■: waxy starch, ●: lactulose. Each data point represents the mean and each vertical bar represents 1 S.D. above the mean for the 3 subjects.

tive energy from the dietary fiber contents obtained by the Prosky method, which does not take the energy produced by fermentation into consideration. If dietary fiber of HB-450 (64.5%: Prosky method) is fermented completely and the remainder (35.5%) is digested completely, the effective energy could be calculated as follows: 64.5(%) × 2 (kcal/g) + 35.5(%) × 4 (kcal/g) = 2.7 (kcal/g).

Effective energy of some RS has been calculated by recent studies:[21,22] retrograded maltodextrin (RS3) is reported to be 2.8 kcal/g (*in vivo*),[21] and high amylose maize (RS2) is 2.7–2.8 kcal/g (*in vitro*).[22] These values agree with the present data. However, the values are differ greatly among the RS tested (2–3 kcal/g) in the previous reports,[23] and thus further individual experiments using many

types of RS will be needed in the future.

Mochida *et al.*[15] have reported that daily ingestion of pasta including 10 g of HB-450 for 9 days resulted in effective energy of 3.1 kcal/g by analysis of the fecal starch. Nakayama *et al.*[24] also reported that effective energy of a heat-moisture-treated high amylose cornstarch (Roadstar®) is estimated as 2.6–2.8 kcal/g by applying the Prosky method to Roadstar®-containing bread. However, little has been reported concerning the metabolism and recommended levels of RS in the diet for Japanese persons.[25]

Corn is considered to be a C4 plant, and it contains a plentiful quantity of ^{13}C because it fixes CO_2 to C4 intermediate substances via the Hatch-Slack pathway. In contrast, C3 plants fix CO_2 to C3 intermediate substances through the Calvin-Benson reaction. Environmental air typically contains 1.1% $^{13}CO_2$ and 98.9% $^{12}CO_2$. Because of differences in the pathways by which C4 and C3 plants fix CO_2, C4 plants have a higher proportion of ^{13}C content.[12] Carbohydrates are completely oxidized to CO_2 and water in the mitochondria.[26] Past research frequently involved the use of starch labelled with ^{13}C chemically (a stable isotope), but the method using cornstarch (starch labelled with ^{13}C naturally) is less expensive.[27] Therefore, analysis of breath $^{13}CO_2$, H_2 and CH_4 would be a useful method to calculate easily, safely and cheaply the effective energy of RS which is originated from a C4 plant.

Although the study reported here was designed to address the calculation of energy for nutrition assessment, a large-scale analysis will be performed to obtain the effective energy of many kinds of RS. This is very important for estimating the daily energy intake in patients with lifestyle-related diseases.

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