Poor Fermentability of “Mekabu” (Sporophyll of Undaria pinnatifida) Alginic Acid in Batch Culture Using Pig Cecal Bacteria

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Summary Sodium alginate, mannuronic acid-rich and guluronic acid-rich fractions were prepared from “Mekabu” (sporophyll of Undaria pinnatifida). The production of short-chain fatty acids such as acetic, propionic and n-butyric acids from these fractions in mini-scale batch culture using pig cecal bacteria was studied, and the gas released from the culture was monitored. The volume of released gas corrected for blank value decreased in the order: glucose (a reference substrate) > guluronic acid-rich fraction > sodium alginate = mannuronic acid-rich fraction. The amounts of short-chain fatty acids produced from three fractions of alginic acid were smaller than that of glucose. These results suggested that alginic acid was poorly fermentable for hindgut bacteria and that its contribution to the host energy pool via microbial metabolism is small.

Key Words pig, large intestine, fermentation, alginic acid, “Mekabu,” gas release, short-chain fatty acids

“Wakame” (Undaria pinnatifida) is an edible brown seaweed found along the coast of Japan. “Mekabu” is the sporophyll formed near the root of “Wakame” at the ripe stage, normally from March to June. The viscous exudate of the “Mekabu” mainly consists of alginic acid.

Alginic acid, a polysaccharide composed of D-mannuronic acid and L-guluronic acid, comprises the cell wall of brown seaweeds. Alginic acid is not only indigestible by human enzymes, but it decreases the digestibility of other nutrients (1) and enlarges the digestive organs (2). There have been some reports on the digestibility and energy availability of seaweed (3) and the microbial degradation of depolymerized alginate (4, 5).

Indigestible polysaccharides can be fermented in the large intestine to produce short-chain fatty acids (SCFA) and gases. Almost all of the SCFA such as acetic, propionic and n-butyric acids produced in the hindgut are absorbed and enter the...
systemic energy pool of the host animal (6). Apart from their energy contribution, SCFA have physiological influences on digestive organs; they stimulate epithelial cell proliferation of the intestine (7), colonic motility (8) and pancreatic secretion (9). Such influences depend on the amounts and kinds of the produced SCFA.

In other words, physiological influences of indigestible polysaccharides seem to be exerted, at least partly, via the production of SCFA. Accordingly, we measured SCFA production from alginic acid fractions prepared from “Mekabu” in a mini-scale batch culture technique using pig cecal bacteria, as the first step to clarify the physiological effects of alginic acid via the production of SCFA. Prior to the measurement of SCFA production, we studied the time-course of gas released from the cultures.

**MATERIALS AND METHODS**

**Materials.** The dried “Mekabu” harvested in Ise City, Mie Prefecture, was used as the starting material. Sodium alginate used as a standard was purchased from Wako Pure Chemical Industries, Osaka, Japan.

**Substrates.**

Preparation of substrates: The ground “Mekabu” (10.0 g) was shaken in 0.2N sulfuric acid (500 ml) overnight. After filtration through two layers of surgical gauze, sodium alginate was extracted by shaking the residue in 500 ml of 1% sodium carbonate (v/v) overnight. After the addition of water (1 to 2 liters), the diluted solution was filtered through filter paper using a Buchner funnel. An equal volume of ethanol was added to the filtrate to allow sodium alginate to precipitate. After vacuum filtration, sodium alginate was washed with ethanol and diethyl ether and dried in a vacuum drier. The dried sodium alginate obtained was 1.4 g/10.0 g “Mekabu.” According to Haug’s method (10), the extracted sodium alginate (1.8 g) was dissolved in water (400 ml) and mixed with a saturated solution of potassium chloride (400 ml). The mixture was separated into guluronic acid-rich (supernatant) and mannuronic acid-rich fractions (precipitate) by centrifuging (20,000 rpm for 30 min). An equal volume of ethanol was added to both fractions and the mixtures were filtered. The precipitates were washed with ethanol and diethyl ether and dried in a vacuum drier (0.7 and 0.4 g).

Purity of extracted sodium alginate: The purity of extracted sodium alginate was checked using HPLC (LC-10A, Shimadzu) equipped with an Asahipak GF-310HQ column (7.5 mm i.d. x 30 cm long, Shimadzu) and ultraviolet spectrophotometer (SPD-10A, Shimadzu) at 210 nm. The column temperature was 40°C and the mobile phase was 5 mM perchloric acid (flow rate 1.0 ml/min). The purity of extracted sodium alginate was 91%.

Mannuronic acid to guluronic acid (M/G) ratio of extracted sodium alginate: The M/G ratio of extracted sodium alginate was determined using HPLC. The 50 mM hydrochloric acid (5 ml) was added to extracted sodium alginate (10 mg) and the glass ampoule was sealed and kept at 100°C for 24 h to hydrolyze the sample.

The content was neutralized with 100 mM sodium carbonate, and analyzed by HPLC in the same condition as above. Sodium alginate was completely hydrolyzed, the M/G ratio of extracted sodium alginate was 0.77, it agreed with that of "Wakame" harvested in spring; 0.75 (11).

Culture apparatus.

Gas release: A 1 ml-scale batch culture technique was used (12). A glass test tube (i.d. 12 mm × depth 105 mm) with a joint stopper was used as the culture vessel. A small amount of marker solution [75% magnesium chloride (w/v) containing 0.05% methyl orange (w/v)] was placed in a 1-ml glass pipet connected to the test tube with Tygon tubing.

SCFA production: Plastic 1.5-ml sample tubes with a snap-close lid were used. A small hole was made at the center of the lid with an injection needle to release gas during incubation.

Preparation of inoculum. Fresh cecal contents of pigs were sampled within 30 min after slaughter at Seki Shokuniku Center in Gifu Prefecture. The cecal contents of three pigs were pooled and diluted with an equal volume of bicarbonate buffer (NaHCO₃, 9.240 g; Na₂HPO₄·12H₂O, 7.125 g; NaCl, 0.470 g; KCl, 0.450 g; CaCl₂·2H₂O, 0.073 g; MgCl₂·6H₂O, 0.087 g per liter) within 3 h after the sampling. The pH of the buffer was adjusted to 7.0 by bubbling with CO₂ gas. The diluted contents were filtered through four layers of surgical gauze, and the filtrate was centrifuged at 200 × g at 4°C for 5 min. The pellets of bacteria were prepared from the supernatant by centrifuging (35,000 × g at 4°C for 15 min).

Preparation of the culture. One gram of bacterial pellet was mixed with 4 ml of bicarbonate buffer (pH 7.0). Each substrate of 0.1 g was dissolved in 5 ml of bicarbonate buffer. Then an equal volume of bacterial suspension and substrate-containing buffer (500 µl each to monitor the gas release, and 50 µl each to measure SCFA production) were transferred into the culture vessel and agitated with a vortex mixer. After displacement of the head space with CO₂ gas, the test tube for monitoring of the gas release was connected to a glass pipet as stated above. Each vessel (in triplicate) of a given substrate was incubated in the water bath at 37°C for 24 h. The head space of sample tubes for the measurement of SCFA production was also displaced with CO₂ gas before the start of incubation.

Measurement.

Gas release: The volume of released gas from the culture was monitored by visual observation of the movement of marker solution in the pipet for 24 h at 1-h intervals, as shown in Fig. 1. We repeated such monitoring three times using inoculi from different pigs. The volumes of released gas were corrected for blank value.

SCFA production: Each culture (in duplicate) of a given substrate was removed from the water bath at 1, 4 and 8 h of incubation. These tubes were immediately immersed into ice-cold water to stop fermentation. These samples were centrifuged at 10,000 rpm for 10 min. The supernatant was used for analysis of SCFA. The measurement of SCFA production was repeated two times using
inoculi from different pigs. SCFA was measured by the internal standard method (13) using HPLC (LC-6A, Shimadzu) equipped with Shim-pack SCR-102H column (8 mm i.d. × 30 cm long, Shimadzu) and an electroconductivity detector (CDD-6A, Shimadzu). The column temperature was 45°C and the mobile phase was 5 mM p-toluene sulfonic acid (flow rate 0.8 ml/min). The detection reagent was 20 mM bis-Tris containing 5 mM p-toluene sulfonic acid and 100 μM EDTA (flow rate 0.8 ml/min, 45°C).

Statistical analysis. Differences between means were tested by Tukey's multiple comparison test after analysis of covariance taking the time effect as the covariate. The difference was considered significant when the probability was smaller than 0.05.

RESULTS

Gas release

Figure 1 shows the cumulative volume of released gas from sodium alginate, the mannuronic acid-rich fraction, guluronic acid-rich fraction and glucose. The significant two-way interaction effect (time × substrate) indicated that the time-course of gas release varied among substrates (p < 0.001). Gas release started within 1 h and ceased after 8 h of incubation with glucose. Gas release started after 10 h of incubation with three fractions of alginic acid and continued for 24 h. The cumulative volume of released gas remained negative at least until 12 h of culture with sodium alginate and mannuronic acid-rich fraction. The volume of released gas corrected for blank value decreased in the order: glucose > guluronic acid-rich fraction > sodium alginate = mannuronic acid-rich fraction (p < 0.001).

Fig. 1. Time courses of gas release from 10 mg of sodium alginate (●), Man-rich fraction (▲), Gul-rich fraction (■) and glucose (○) in batch culture using pig cecal bacteria after the subtraction of blank value. Each point represents the mean of three replications using inoculi from different pig groups.
Table 1. SCFA productions from 10mg of each alginic acid fraction in batch culture using pig cecal bacteria.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Sodium alginate</th>
<th>Man-rich fraction</th>
<th>Gul-rich fraction</th>
<th>Glucose</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>10.2</td>
<td>9.7</td>
<td>10.5</td>
<td>25.1</td>
<td>13.3</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>4.8</td>
<td>4.3</td>
<td>4.7</td>
<td>23.5</td>
<td>5.7</td>
</tr>
<tr>
<td>n-Butyric acid</td>
<td>1.2</td>
<td>1.3</td>
<td>1.7</td>
<td>3.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Total</td>
<td>16.2</td>
<td>15.3</td>
<td>16.9</td>
<td>51.7</td>
<td>20.5</td>
</tr>
</tbody>
</table>

After 1 h of incubation (µmol)

| Acetic acid        | 13.4            | 15.5              | 14.7              | 48.9    | 15.7  |
| Propionic acid     | 5.4             | 6.3               | 6.0               | 47.6    | 6.5   |
| n-Butyric acid     | 1.9             | 2.5               | 1.9               | 4.8     | 2.7   |
| Total              | 20.7            | 24.3              | 22.6              | 101.3   | 24.9  |

After 4 h of incubation (µmol)

| Acetic acid        | 20.8            | 17.3              | 21.2              | 58.2    | 24.1  |
| Propionic acid     | 6.9             | 6.5               | 7.1               | 58.2    | 8.0   |
| n-Butyric acid     | 3.3             | 2.8               | 3.2               | 5.1     | 2.8   |
| Total              | 31.0            | 26.6              | 31.5              | 121.5   | 34.9  |

After 8 h of incubation (µmol)

The values are means of duplicated measurements using inoculi from different pig groups.

**SCFA production**

SCFA production from sodium alginate, the mannuronic acid-rich fraction, guluronic acid-rich fraction, glucose and blank after 1, 4 and 8 h of incubation are shown in Table 1. Cultures with glucose produced a larger amount of acetic, propionic and n-butyric acids than those with sodium alginate, mannuronic acid-rich or guluronic acid-rich fractions ($p < 0.001$). The amounts of each SCFA produced in cultures with sodium alginate, mannuronic acid-rich and guluronic acid-rich fractions decreased in the order: acetic acid > propionic acid > n-butyric acid at all time points. The molar ratio of acetic acid : propionic acid : n-butyric acid was approximately 6 : 3 : 1 for the three fractions of alginic acid, while that for glucose was approximately 10 : 10 : 1.

**DISCUSSION**

The maximums of cumulative volume of released gas and amount of total SCFA production from glucose (10mg) were 1.17ml and 121.5 µmol after 8 h of incubation, respectively. These values agreed with previous reports (12, 14), indicating the methodological validity of the present study.

The use of pig bacteria for a model study aiming for human nutrition was carefully discussed and validated (12); the pig is an omnivore having body weight
comparable to that of humans, and intestinal microflora of the pig resemble those of humans. It is also noteworthy that the use of pig bacteria is safer than the use of human bacteria, because the latter always have theoretical possibility of transfection of pathogenic viruses such as hepatitis virus. Further, the production of SCFA mainly depends on the nature and the entry rate of the substrate, rather than the host species (15). This again supports the use of pig cecal bacteria as a model for the human hindgut ecosystem.

The volume of released gas correlated positively and linearly with the total amount of SCFA produced from various indigestible saccharides (12, 14). However such as correlation was not found in the present study, probably due to the poor production of SCFA from alginic acid.

Inter-group comparison of SCFA production (Table 1) indicated that the addition of alginic acids, irrespective of the fraction, did not increase the SCFA production from the blank level. This and the negligible gas release from cultures with alginic acids suggest that alginic acid is a hardly fermentable substance. The time-dependent increase in the SCFA concentration in cultures with alginic acids can be attributed to the microbial degradation of the inoculum itself (lysis), because the molar proportion of SCFA was between those of alginic acids and the blank group (Table 1). Thus, the microbial SCFA production from alginic acid should be negligible. Accordingly, we cannot expect energy contribution and physiological influences via hindgut SCFA production from alginic acid at least under the conditions of the present study. The use of inoculi from many different pigs and the reproducibility of the results further validate the above statement.

We used pig cecal bacteria in this study without preliminary adaptation to alginic acid. The adaptation of bacteria to alginic acid would increase the microbial degradation of it. Kimura et al. reported that the human intestinal bacteria degraded alginic acid almost completely in vitro, but this result was not consistent with that observed in vivo (16). These reports should be taken into account, when the present results are extrapolated to humans in vivo.

REFERENCES
