Growth-promoting effects of hydrolyzed hen egg white on Lactobacillus and Bifidobacterium sp.

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SUMMARY

To evaluate growth effects of an H-8; hydrolyzed hen egg white preparation, on lactobacilli and bifidobacteria, we used Lactobacillus delbrueckii subsp. bulgaricus SU-3, L. casei SBR 1202, Bifidobacterium breve SBR 3213, B. adolescentis N-3 and B. adolescentis N-4. It was revealed that 0.6% H-8 in skim milk has significant growth-promoting effects on these strains. Fermented skim milk with H-8 by each tester strain had significantly higher antimutagenicity towards Trp-P-1 than these without H-8. Furthermore, male Wister rats at 6 weeks old were fed the diets with 10% H-8 to evaluate effects on their intestinal flora. The number of aerobes appeared on Standard plate count agar and coliforms in their feces were smaller than that from rats fed diets without H-8. Contrary, the number of lactic acid bacteria in the feces showed a significant increase with dose-relating manner. These results indicated that H-8 could be one of prebiotics.

INTRODUCTION

Lactobacillus and Bifidobacterium sp. are normal residents of the complex ecosystem of the gastrointestinal tract¹, and are expected to exert health-promoting effects on humans and animals. These effects include inhibitions of infection with pathogenic microorganisms and their functions²³.
and antimutagenic and anticarcinogenic activities\(^{4,8}\). Probiotics, defined as living microbes in food supplements, benefit the host by improving microbial balance in its intestine\(^6\). In contrast, prebiotics are non-digestible nutritional compounds, such as peptides or oligosaccharides, that selectively stimulate the growth of endogenous Lactobacillus and Bifidobacterium sp. to improve the health of the host\(^7\). Foods in any form that reaches the colon, e.g. non-digestible carbohydrates, some peptides and proteins, as well as certain lipids, are candidates for a prebiotics. The prebiotics approach advocates administration of non-viable entities and aims to overcome the survival problems of probiotics in the upper gastrointestinal tract\(^8\).

Carcinogenicity has always been correlated with modification of gut bacterial activities\(^9\). The intestinal microbiota constitutes a complex ecosystem of a large variety of bacteria. This microbiota may affect the host either positively or negatively\(^8\), thus altering the composition of microflora in a beneficial way may improve the health of a host. Lactobacillus and Bifidobacterium sp. have been considered potentially useful in this respect. H-8, which is a hydrolyzed hen egg white and that include peptide with a molecular weight of 10kDa\(^8\), may act as a growth promoter for Lactobacillus and Bifidobacterium sp.. And it would be a cost-effective source of peptides, so it would be useful for prebiotics. Based on the functional food concept, probiotic and prebiotic approaches are important issues. We studied the role of a hydrolyzed hen egg white preparation, namely the H-8, in growth-promoting effects on Lactobacillus and Bifidobacterium sp..

MATERIALS AND METHODS

Bacterial cultures
Lactobacillus casei SBR 1202, and Bifidobacterium breve SBR 3213 were obtained from Kagome Labio Co. Ltd. (3-45, Kokihigashi, Komaki, Aichi, 485-0059, Japan). B. adolescentis N-3, and B. adolescentis N-4 were obtained from Harada milk Co. Ltd. (3040 Michigane, Tsubame, Niigata, 959-1200, Japan). Lactobacillus delbrueckii subsp. bulgaricus SU-3 was a stock culture of Shinshu University (8304 Minamiminowa, Nagano, 399-4598, Japan). L. casei SBR 1202 and B. breve SBR 3213 were used as probiotics\(^{10,12}\). Lactobacillus sp. were basically incubated in skim milk medium (Snow Brand Products Co. Ltd., Tokyo, Japan). Bifidobacterium sp. were basically incubated in skim milk medium including 0.5% yeast extract (Nacalai tesque, Kyoto, Japan). All colonies appeared on MRS plates (OXOID, Hampshire, UK) were counted as colony-forming-units (cfu).

Salmonella typhimurium SD 510, a streptomycin-dependent derivative of S. typhimurium TA 98, was maintained on SM 20 broth, an Oxoid nutrient broth (OXOID, Hampshire-UK) fortified with 20 \(\mu\)g/ml streptomycin (Meiji Seika Co., Tokyo-Japan).

Preparation of H-8 from hydrolyzed hen egg white fraction
Hen egg white was separated from whole egg, homogenized, sterilized at 60°C for 3.5 minutes, and adjusted to pH 7.4 with 10% citric acid solution. This preparation was inoculated with Saccharomyces cerevisiae IFO 1950 (1% (w/w) in the final concentration) and incubated at 30°C for 3h to remove the sugar fraction, then digested with actinase AS (0.21g/kg egg white in the final concentration; Nacalai tesque, Kyoto, Japan) at 50°C for 24h. Finally, it was spray-dried at 140°C to produce H-8. H-8 was stored at 4°C prior to experimental use.

Preparation of mutagens
Amino acid pyrolyzate (3-amino-1, 4-dimethyl-5 H-pyrido [4,3-b] indole; Trp-P-1, Wako Pure Chemical, Tokyo, Japan) was used as a mutagen. Distilled water was used as a solvent.

Antimutagenicity test
The antimutagenicity of cultures made through fermentation of skim milk medium with or without 0.6% H-8 fermented by L. casei SBR 1202 B. breve SBR 3213 or L. delbrueckii subsp. bulgaricus SU-3 toward Trp-P-1 was assayed using S. typhimurium SD 510\(^{11+}\) for 24h. S. typhimurium SD 510 was incubated with SM 20 broth in a shaking water bath at 37°C to an optical density of
1.3 at 660 nm (5.0 × 10⁶ cfu/ml), then diluted with 0.05 M phosphate buffer (pH 6.8) to 5.0 × 10⁵ cfu/ml. The Oxoid nutrient broth plates were previously applied by Conradi stick with 100 µl portion of the dilution. And each of 200 µl fermented cultures was mixed with 100 µl of 100 µg/ml Trp-P-1 and was pre-incubated at 37°C for 30 minutes, then 40 µl of the mixture was discharged on a sterile 8 mm-paper disc placed on the Oxoid plate. The plates were incubated at 37°C for 48h and the revertant colonies were counted. Positive control consisted of 100 µl of 100 µg/ml Trp-P-1 and 200 µl sterile distilled water instead of each fermented cultures. Negative control consisted of 100 µl distilled water instead of the mutagen and 200 µl of each fermented cultures. The number of spontaneous revertants obtained from the negative control was subtracted from that of each tested sample to obtain the number of induced revertants. Results are indicated as the number of revertants per plate.

Antimutagenicity was estimated by measuring the decrease in the number of mutations induced by Trp-P-1, indicated as inhibition percentages (PI) and calculated by the formula: PI=(1-(number of revertants on test plate)/(number of revertants on positive control plates))×100.

Animals

Twenty male Wistar rats at 6 weeks old weighing 120-150g (SLC, Kyoto, Japan) were housed in individual metabolic cages at 22-24°C, relative humidity of 55-65%, and a 12 hours light/dark cycle. The animal care was in accordance to the guidelines for Animal Experimentation of the School of Medicine, Shinshu University. All rats were used after acclimatization feeding a commercially available basal chow diet (Clea Japan Inc., Osaka, Japan) ad libitum for 5 days.

Five animals were randomly assigned to each group. A group was fed a normal diet (above-mentioned basal chow diet), and the other groups were fed a normal diet supplemented with 1%, 5%, and 10% H-8, respectively for 5 days. After the supplementation of H-8, a normal diet was given ad libitum for the next three days. Water was given ad libitum during the experiment. Body weight of each rat was measured before grouping and on the completion of the experiment.

Fecal Microbial Analysis

All fecal samples were collected freshly by gentle squeezing rectums of the rats. The fecal pellets were immediately put into tubes kept in anaerobic jars and analyzed in 30-60 minutes after the collection. Anaerobic conditions were kept during the analysis as far as possible. Following homogenization, a series of 10-fold dilutions of the specimens was made in a sterilized phosphate buffer. Three kinds of plates were made for each fecal sample; Standard plate count agar (OXOID, Hampshire, UK) for anaerobes and aerobes, Desoxycholate agar (Nissui, Tokyo, Japan) for coliforms, and MRS agar for Lactic acid bacteria (LAB). Plates of anaerobes and LAB were incubated in an anaerobic chamber (BBL Gas Pak anaerobic jars, Becton Dickinson Co., Franklin Lakes-NJ) at 37°C for 3 days.

Statistical Analyses

Obtained results were subjected to Student t-test using Microsoft Excel Ver. 6.0 (Microsoft Corporation, Redmond, WA, USA) Williams multiple comparison, and Cochran-Armitage trend test.

RESULTS AND DISCUSSION

Hen egg white ovomucin is a fibriform and highly viscous glycoprotein, which accounts for approximately 3.5% of egg white protein. It consists of an α-subunit (apparent molecular mass (AMM) ~200kDa) containing 10-15% carbohydrate and a β-subunit (AMM ~400kDa) containing 50-65% carbohydrate, with a macromolecular structure stabilized by disulfide bonds between the subunits. H-8 is prepared from hen egg white treated with Saccharomyces cerevisiae IFO 1950 and Actinase A S™. It contains ovomucin-derived fraction. Tsuge et al. derived glycopeptide with molecular of 10 kDa from ovomucin according to same methods of preparation of H-8™. H-8 significantly enhanced the growth of the Bifidobacterial tester strains in the skim milk for both 12h and 24h incubation.
times (Figures 1, 2, and 3). Growth of B. breve SBR 3213 was promoted by more than one log cycle. And that of B. adolescentis N-3 and B. adolescentis N-4 were promoted by less than one log cycle. Growth of L. casei SBR 1202 was promoted by II-8 after 24h incubation (Fig.4). These results indicated that H-8 might be the nutritive substance for these strains. Gomes et al. reported that the growth of Bifidobacterium and Lactobacillus were enhanced by milk hydrolyzates. As H-8 was also prepared by enzyme-digestion, it includes not only ovomucin-derived fraction but also albumin-derived fraction, it might be easy to use as nitrogen source for these strains. Yeast extract recognized as a growth promoter to bifidobacteria was also applied to prepare H-8. However the amount of carried over of yeast extract in H-8 was a very small (lower than 1.8% (w/w) of the total yeast extract for bifidobacteria or lower than 1.5% (w/w) in H-8), thus it would not work as a growth promoter.

Skim milk fermented by L. casei SBR 1202, B. breve SBR 3213, or L. delbrueckii subsp. bulgaricus SU-3 inhibited the mutagenicity of Trp-P-1 by 52.4%, 38.61%, and 65.86% in vitro, respectively (Table 1). With 0.6% H-8, skim milk fermented by L. casei SBR 1202, B. breve SBR 3213, or L. delbrueckii subsp. bulgaricus SU-3 showed significant antimutagenicity towards Trp-P-1 by 81.09%, 54.04%, and 84.42%, respectively (Table 1). Skim milk including H-8 (0.3%, 0.6% and 1.0%) fermented by L. casei SBR 1202 inhibited the mutagenicity of Trp-P-1 by 76.97%, 81.09%, and 82.13%, respectively with dose-dependent manner. Sreekumar et al. reported that mutagens were combined with Lactobacillus gasseri and Bifidobacterium longum cells, thus antimutagenicity by the fermented cultures were indicated that it is due to the increase of the number of the tester strains by H-8. Carcinogenicity as well as mutagenicity has been correlated to the modification of intestinal bacteria. Escherichia coli and Bacteroides have been reported to produce enzymes, such as β-glucuronidase and azoreductase, which convert pro-carcinogens into carcinogens in the intestinal tract. If probiotics would consume H-8 in bowels, they should inhibit the growth of the bacteria producing these enzymes.

In this study, the number of fecal coliforms appeared on Desoxycholate agar and aerobes appeared on Standard plate count agar significantly decreased in the fecal samples of rats fed with 10% H-8 (Fig.5). Coliform and aerobes in them significantly showed decreasing with dose-relating manner, the other, lactic acid bacteria in the fecal samples showed significant increase with a dose-relating manner. In consequence, H-8 administered through the diet supported the growth of intestinal lactic acid bacteria, which resulted to reduce the number of intestinal coliforms and aerobes. Prebiotics are defined as non-digestible food ingredients (oligosaccharides, dietary fiber, starch, etc.) that beneficially affect on the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already established in the colon, thus in effect improve host health. H-8 might include a non-digestible food ingredient because apparent molecular mass of ovomucin-derived fraction of H-8 is respectable big as mentioned above and it was residue of reaction which was digested by Actinase AS. H-8 included a non-digestible ovomucin-derived fraction, and showed growth-promoting effect on intestinal microflora such as Lactobacillus and Bifidobacterium sp. in accordance with prebiotic criteria. Accordingly, H-8 would be correlated to the definition that prebiotics is a non-digestible nutritional material that selectively stimulates the growth of endogenous microflora to improve the health of the host.

Lactic acid bacteria are recognized as beneficial bacteria, and various microbial strains, mostly from the genera Lactobacillus and Bifidobacterium, have been used as probiotic agents for decades. In addition, it is also widely accepted that the gut microbiota as a whole plays a pivotal role in maintaining gut health and protecting the host from infections. Therefore, maintaining a good balance of gut microbiota is of importance to the health of the host. Yukuchi et al. classified that the basic physiological functions of the probiotic microorganisms in the digestive tract are as follows: (1) promotion of growth and digestion, (2) settling effects on the gastrointestinal tract, (3)
Fig. 1. Effect of addition of 0.6 % H-8 to skim milk on the viable count of *Bifidobacterium breve* SBR 3213

Both of the skim milk with and without H-8 were incubated for 12 and 24 hours and viable count was made as colony-forming-unit (cfu) on MRS plate. Data bars represent the mean ± s.e.
Filled column: The number of SBR 3213 incubated in skim milk with 0.5% yeast extract and 0.6% H-8.
Unfilled column: The number of SBR 3213 incubated in skim milk with 0.5% yeast extract and without H-8.
*Statistically significant difference (P<0.05) between with and without H-8.

Fig. 2. Effect of addition of 0.6 % H-8 to skim milk on the viable count of *Bifidobacterium adolescentis* N-3

Both of the skim milk with and without H-8 were incubated for 12 and 24 hours and viable count was made as colony-forming-unit (cfu) on MRS plate. Data bars represent the mean ± s.e.
Filled column: The number of N-3 incubated in skim milk with 0.5% yeast extract and 0.6% H-8.
Unfilled column: The number of N-3 incubated in skim milk with 0.5% yeast extract and without H-8.
*Statistically significant difference (P<0.05) between with and without H-8.

Fig. 3. Effect of addition of 0.6 % H-8 to skim milk on the viable count of *Bifidobacterium adolescentis* N-4

Both of the skim milk with and without H-8 were incubated for 12 and 24 hours and viable count was made as colony-forming-unit (cfu) on MRS plate. Data bars represent the mean ± s.e.
Filled column: The number of N-4 incubated in skim milk with 0.5% yeast extract and 0.6% H-8.
Unfilled column: The number of N-4 incubated in skim milk with 0.5% yeast extract and without H-8.
*Statistically significant difference (P<0.05) between with and without H-8.

Fig. 4. Effect of addition of 0.6 % H-8 to skim milk on the viable count of *Lactobacillus casei* SBR 1202

Both of the skim milk with and without H-8 were incubated for 12 and 24 hours and viable count was made as colony-forming-unit (cfu) on MRS plate. Data bars represent the mean ± s.e.
Filled column: The number of SBR 1202 incubated in skim milk with 0.5% yeast extract and 0.6% H-8.
Unfilled column: The number of SBR 1202 incubated in skim milk with 0.5% yeast extract and without H-8.
*Statistically significant difference (P<0.05) between with and without H-8.
Table 1. Effect of addition of H-8 to skim milk fermented with single strain of *Lactobacillus* and *Bifidobacterium* on its antimutagenicity toward Trp-P-1 (100 μg/mL)

<table>
<thead>
<tr>
<th>Strains</th>
<th>None (Water) (a)</th>
<th>Positive Control (Trp-P-1 only) (b)</th>
<th>Treatment (c)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus casei</em> 1202 Rolly C</td>
<td>16±10</td>
<td>16±10</td>
<td>16±10</td>
<td>52.40</td>
</tr>
<tr>
<td><em>L. casei</em> 1202 Rolly C + 0.3% H-8</td>
<td>598±50</td>
<td>598±50</td>
<td>598±50</td>
<td>76.97</td>
</tr>
<tr>
<td><em>L. casei</em> 1202 Rolly C + 0.6% H-8</td>
<td>598±50</td>
<td>126±10</td>
<td>120±10</td>
<td>81.09</td>
</tr>
<tr>
<td><em>L. casei</em> 1202 Rolly C + 1.0% H-8</td>
<td>598±50</td>
<td>120±10</td>
<td>82.13</td>
<td></td>
</tr>
<tr>
<td><em>Bifidobacterium breve</em> 3213 Rolly B</td>
<td>7±7</td>
<td>688±40</td>
<td>425±31</td>
<td>38.61</td>
</tr>
<tr>
<td><em>B. breve</em> 3213 Rolly B + 0.6% H-8</td>
<td>7±7</td>
<td>688±40</td>
<td>320±21</td>
<td>54.04 <em>c</em></td>
</tr>
<tr>
<td><em>Lactobacillus delbrueckii</em> subsp. bulgaricus SU-3</td>
<td>10±4</td>
<td>716±23</td>
<td>251±23</td>
<td>65.86</td>
</tr>
<tr>
<td><em>L. delbrueckii</em> subsp. bulgaricus SU-3 + 0.6% H-8</td>
<td>10±4</td>
<td>716±23</td>
<td>120±16</td>
<td>84.42 <em>c</em></td>
</tr>
</tbody>
</table>

a: Inhibition(%)= [−((c)−(a))−((b)−(a))] ×100
b: P<0.05 (subjected to Williams multiple comparison)
c: P<0.05 v.s. skim milk fermented with corresponding strain without H-8 (subjected to Student's t-test)

Fig. 5. Effect of H-8 on the fecal flora of male Wister rats.

Rats were fed with normal diet (group A), containing 1% H-8 (group B), containing 5% H-8 (group C), or containing 10% H-8 (group D). Their feces in the rectal area were examined on day 5 after the feeding. Four kinds of bacteria, such as aerobes appeared on Standard plate count agar (■), coliform appeared on Desoxycholate agar (○), anaerobes appeared on Standard plate count agar (□), and Lactic acid bacteria *Lactobacillus* appeared on MRS agar (▲) were examined in their faces.

*Significantly different (P<0.05) from the control group (group A).
a: Showed significant trend (P<0.05)
improvement of bowel movement, (4) anticancer action, (5) lowering of blood cholesterol, and (6) stimulation of the immune system in the gut. Among these characteristics, *B. breve* SBR 3213 and *L. casei* SBR 1202 have already been known to improve bowel function in humans. Mori et al. 12 studied the effects of milk products fermented with *B. breve* SBR 3213 and *L. casei* SBR 1202 in 61 healthy female volunteers (average age 20.0 ± 2.8) and reported that defecation frequency and defecation volume increased compared to the pre-administration period for the constipated volunteers.

There are a few reports that have analysed the suppressive effect of prebiotics on the development of cancer in model systems using rats and chemical carcinogens. In an experiment executed to monitor the development of colorectal cancer induced by 1,2-dimethylhydrazine in rats, fully-fermentable β-gluc-oligosaccharides appeared to be highly protective. In this respect, it would be interesting to investigate H-8’s ability to help suppress the development of cancer. Because the present results indicated that H-8 has a significant effect on the growth of *Lactobacillus* and *Bifidobacterium* sp.. Both of them are suggested as adsorbent toward mutagen, like Trp-P-1. Modification of the microflora by prebiotics has also been a rational approach for controlling intestinal inflammation. Therefore, the possible mechanism for the enhancing properties of H-8 needs to be studied further.

References


18) M. David Collins, and Glenn R. Gibson: Probiotics,


卵白タンパク質由来酵素分解物による乳酸桜菌並びにビフィズス菌に対する増殖促進効果

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卵白タンパク質由来酵素分解物である H-8 が、乳酸桜菌とビフィズス菌の増殖に与える影響を調べた。用いた菌株は Lactobacillus delbrueckii subsp. bulgaricus SU-3, L. casei SBR 1202, Bifidobacterium breve SBR 3213, B. adolescentis N-3 と B. adolescentis N-4 であった。その結果、0.6%H-8 を添加したスキミルクにはこれらの菌株に対して有意な増殖促進効果が認められた。一方で H-8 を添加し、それぞれの菌で培養したスキミルク培養液は、H-8 を添加しなかったスキミルク培養液に比べて、Trp-P-1 に対する抗異常原性が有意に上昇した。さらに 6 週齢の Wister 系雄ラットに 10%H-8 を含む断食餌を給餌し、H-8 の腸内菌叢に与える影響を調べた。その結果、H-8 を給餌したラットの糞便中の標準栄養栄養中由来の好気性菌数と大腸菌数は、H-8 を含まない断食餌を給餌した群の糞便中のそれぞれの菌数と比較して有意に減少した。一方で乳酸菌の菌数は用量依存的に増加した。これらの結果より、H-8 にはプレバイオティクスとしての可能性が示唆された。

Key Words : H-8 from hydrolyzed hen egg white, prebiotics, Lactobacillus, Bifidobacterium, antimutagenicity