Complementary Effects of Bifidogenic Growth Stimulators and Ammonium Sulfate in Natural Rubber Serum Powder on Bifidobacterium bifidum

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Natural rubber serum powder, rich in crude protein and carbohydrates, had a strong growth-stimulating activity for Bifidobacterium bifidum JCM 1254, which was unable to grow in a fully synthetic medium, B12 assay medium. Natural rubber serum powder was fractionated by ultrafiltration (molecular weight cutoff 1000). The active ultrafiltrate was further concentrated and desalted with an adsorptive microconcentrator, which adsorbs virtually all amino acids and peptides. Through this purification step, it was found that the adsorbed fraction obtained did not stimulate growth independently but acted complementarily with a small amount of ammonium sulfate. The adsorbed fraction was subsequently analyzed on reversed-phase high pressure liquid chromatography, and the activities of the eluates were measured on B12 assay medium with ammonium sulfate. Consequently, it was proved that several peptide ingredients in the adsorbed fraction increased the growth of B. bifidum.

Key words: natural rubber serum powder; Bifidobacterium; bifidogenic growth stimulator; ammonium sulfate

Bifidobacteria, like the lactic acid bacteria, are very beneficial to the health of mankind. The most important benefits on the host health are inhibition or displacement of undesirable microorganisms, elimination of procarcinogens, immunomodulation, and vitamin production. As a result, they are often used as food supplements and in milk fermentation. However, biomass production is slow because these microorganisms require a growth-stimulating factor, bifidogenic growth stimulator (BGS) which is usually absent even in fully synthetic media composed of sugars, vitamins, and nucleic and amino acids. Therefore, several investigations have been reported on a number of potential sources of BGSs. Poch and Bezkorovainy showed the availability of bovine casein digest and yeast extract as BGSs in a fully synthetic medium similar to the well-known Norris medium. Moreover, Ibrahim and Bezkorovainy investigated the growth-stimulating activity of various organic compounds for B. longum by using B12 assay medium, which alone permitted only limited bifidobacterial growth. However, there are only a few reports on completely identified BGSs to date.

Natural rubber serum powder (NRSP) is a natural rubber waste, rich in crude protein and carbohydrates, produced during the manufacture of latex rubber. Several attempts to use NRSP as a newly fermentable substrate have been done in our laboratory as a means of reducing its polluting effect on the environment. We have observed that NRSP has a strong growth-stimulating activity for a wide range of bifidobacteria of human origin. In addition, Oiki et al. investigated the effects of NRSP on B. bifidum in detail using bifidobacterium medium and/or the minimal medium without organic nutrient. NRSP showed synergistic effects with various organic nutrients in a similar manner to casein, which is composed of many kinds of peptides. However, these nutrients had different concentration-related effects on the growth of B. bifidum. Further investigating into the nature of NRSP, the complexity of the experimental media appears to make it difficult to clearly understand the nutritional effects of NRSP on B. bifidum, and to distinguish whether the effect of NRSP is attributed to a simple nutritional source or BGS. Furthermore, we have observed a lower sensitivity of cells to the growth-stimulating activity of BGSs when partially purified BGSs from NRSP was added to bifidobacterium medium, which alone could easily lead to much cell proliferation. To solve these problems, the use of a chemically defined medium mentioned above is necessary. In this paper, we attempted to prove the growth-stimulating effects of NRSP by using B12 assay medium, and to isolate the active ingredients from NRSP that act as BGSs.

Materials and Methods

Microorganism. B. bifidum JCM 1254, of human origin, was purchased from the Japan Collection of Microorganisms, Wako.

Natural rubber serum powder (NRSP). NRSP was provided by Nakanihon Air Service Co., Ltd. (Nagoya). NRSP used in this study was the spray-dried product of natural rubber serum imported from Malaysia. It was composed of many kinds of amino acids, peptides, inorganic salts, and so on. The detailed composition of NRSP was described previously.

Bioassay. The microorganism was grown in anaerobic jars (GasPak, BBL Microbiology Systems, Cockeysville, MD, U.S.A.) in thioglycolate (TGC) medium without dextrose (Difco Laboratories, Detroit, MI, U.S.A.) and stored at 5°C. The stored culture was first

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cultured in the bifidobacterium medium at 37°C for 24 h. The culture broth was centrifuged at 8,000 \times g for 10 min. The precipitated cells were washed two times and suspended with a sterile solution of KCl (1.08%). The basal medium used for a bioassay was B12 assay medium (Difco). The assay medium (3.6 ml) was mixed with 0.4 ml of samples and then inoculated with 5% of the prepared inoculum. The control contained 0.4 ml of water instead of the samples. Culture was done under anaerobic conditions at 37°C. The extent of growth was measured by the absorbance at 562 nm (A562) at various times. The growth experiments were done in duplicate or more each time, and resulting values were averaged in each experiment.

**Purification of BGSs.** NRSP was ultrafiltered with a membrane of molecular weight cutoff (MWCO) 1000 (Amicon, Inc., Beverly, MA, U.S.A.). The ultrafiltrate obtained was transferred into an adsorptive microconcentrator (Microcon-SCX, Amicon) and centrifuged at 1,200 \times g for 1 min to remove low-molecular-weight contaminants from the samples. The components adsorbed on the column were eluted with 1.4 N NH4OH/MeOH desorption reagent by centrifugation at 14,000 \times g for 15 sec. The eluate was dried with a Speed Vac Concentrator (Savant Instruments, Inc., U.S.A.) and dissolved in water. The prepared samples were separated by a reversed-phase HPLC on a Daisopak SP-120-5-ODS-BP column (Daiso Co., Ltd., Osaka, Japan; 25 cm \times 4.6 mm inner diameter) and eluted with a linear CH2CN gradient (5-40%, 35 min) in water at a flow rate of 0.5 ml/min, while monitoring the absorbance at 210 nm. The isolated fractions were then collected and dried with the Speed Vac Concentrator.

**Results**

**Effects of NRSP in B12 assay medium**

Although *B. bifidum* could not be grown in B12 assay medium at all, the addition of 1% (w/v) NRSP to the medium greatly increased its growth (Fig. 1). This result indicates the higher sensitivities of cells to the growth-stimulating activity of NRSP on B12 assay medium, the cell growth being slower compared to it in the bifidobacterium medium. NRSP contains ammonium sulfate, the concentration of which was found to be about 25% (w/w-NRSP). Since ammonium sulfate is known to be one of the major nitrogen sources of bifidobacteria, this inorganic salt in NRSP was considered to have some of the growth-stimulating activity of NRSP as a contaminant. Actually, ammonium sulfate aided the growth of *B. bifidum* on a nutritional medium, which was inferior to the effect of NRSP. Therefore, the effects of ammonium sulfate at the concentration corresponding to that contained in NRSP on *B. bifidum* was assessed in this experiment. As shown in Fig. 1, the growth of the test strain was slightly affected by the addition of 0.25% ammonium sulfate to the medium. These results indicated that biologically active ingredients other than ammonium sulfate, namely BGSs, would probably be the main active principles of NRSP and that B12 assay medium was useful for evaluating the growth-stimulating activity of BGSs. In the process of purifying the BGSs from NRSP, thus, the growth-stimulating activity was defined as the absorbance of 562 nm after cultivation for 40 h.

**Partial purification of BGSs from NRSP**

As the first purification step, we fractionated NRSP by using ultrafiltration (MWCO 1000) to estimate the molecular size of the BGSs in NRSP. As shown in Fig. 2, the ultrafiltrate obtained had an activity almost equal to that of untreated NRSP, but the retained fraction hardly showed any growth-stimulating effect. This suggested that low-molecular-weight material, less than 1000 Da, would be responsible for the growth-stimulating activity of NRSP.

The adsorptive microconcentrator contains a strong cation exchange membrane which adsorbs virtually all amino acids and peptides. This device was used to concentrate peptides and to desalt the ultrafiltrate. However, the adsorbed fraction obtained did not have the complete growth-stimulating effect, even when the concentration of the fraction in the medium was increased (Fig. 3). Thus, despite a lack of the growth-stimulating activity in ammonium sulfate (Fig. 1), the activity of the ultrafiltrate was completely lost by desalting. To solve this contradiction, we assumed that the ammonium sulfate removed could be one of the key components for the expression of the activity of the adsorbed fraction. Figure 3 shows that the adsorbed fraction had a growth-stimulating activity for *B. bifidum* in a dose-dependent manner in the presence of 0.25% ammonium sulfate. This result showed the presence of certain growth stimulators in the adsorbed fraction. The activity of the adsorbed fraction, which was supplemented with ammonium sulfate, at the concentration corresponding to that contained in the ultrafiltrate was a little lower than that of the ultrafiltrate itself (The former value was 0.55 in Fig. 3, and the latter was 0.92 in Fig. 2). Thus, we established a successful bioassay system to...
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To rapidly isolate the BGSs from NRSP, the adsorbed fraction was directly analyzed on reversed-phase HPLC. The chromatogram is shown in Fig. 4. The eluates were broadly fractionated into four fractions, and the growth-stimulating activity of each fraction was measured on the B12 assay medium with 0.25% ammonium sulfate. Consequently, each fraction except for fraction A showed a growth-stimulating activity which was characteristic to that fraction (Table 1). The activity of the mixture of all the HPLC fractions was nearly identical with that of the adsorbed fraction. Since all the active fractions showed a positive ninhydrin-reaction test, the active ingredients in these fractions appeared to be amino acids and/or peptides. However, the addition of a mixture of 20 kinds of amino acids could not stimulate the growth of the test strain on B12 assay medium (data not shown). This indicated that the peptide structure of the substances could be solely responsible for their functional activity.

**Discussion**

Some growth stimulators for other microorganisms, for example, *Klebsiella* and *Lactobacillus*, have been isolated from proteinaceous compounds as a single compound.\(^{1,2}\) However, in this study, composite growth factors existed in the adsorbed fraction. Interestingly,
the high-molecular-weight fraction obtained by ultrafiltration also had strong growth-stimulating effects when 0.25% ammonium sulfate was added. The growth-stimulating activity of the low-molecular-weight fraction was 2.11 times of that of the high-molecular-weight fraction. Therefore, we focused on the purification of the BGSs in the low-molecular-weight fraction. Poch and Bezkorovainy\(^{23}\) have tried to isolate BGSs from casein enzymatic hydrolyzates. Although they found that sulfhydryl groups were indispensable for the expression of the activity of casein enzymatic hydrolyzates, several synthetic peptides including cysteine and/or cystine had no growth-stimulating activity. Furthermore, dispersal over several fractions and decrease in the growth-stimulating activity were observed during the purification of BGSs from casein enzymatic hydrolyzates. These results were similar to those obtained in our experiment. Possibly, BGSs derived from proteinaceous compounds could stimulate the growth of \textit{Bifidobacterium} synergistically. Regardless of the number of active peptides in the compounds, the importance of peptide structure has been emphasized because of the unique action mechanism, which can not be explained as the effects of simple amino acid suppliers. Zhao \textit{et al.}\(^{22}\) have supposed that the peptides target periplasmic binding proteins or their peptide permeases of Gram-negative bacteria, resulting in the stimulation of a particular peptide transport system dependent on peptide structure. The activities of four fractions obtained with reversed-phase HPLC were different to some extent. This result indicated the preferential use of the BGSs by \textit{B. bifidum} as the specificity of milk peptide use observed in \textit{Lactococcus lactis}.\(^{24}\) Further investigation into the structure-activity relationships of the BGSs should give some clues to clarify the complexity of demand for nutrients, which is responsible for the limited bifidobacterial growth.

Oiki \textit{et al.}\(^{19}\) showed that NRSP stimulated the growth of \textit{B. bifidum} in a similar manner to casein on the basis that both could be used not only as a growth stimulator but also as a source of nitrogen by \textit{B. bifidum} on the minimal bifidobacterium medium. However, the complete removal of ammonium sulfate from NRSP revealed that the BGSs in NRSP could not act as a simple nitrogen supplier independently but express the activity through the complementary effects with inadvertently contaminating ammonium sulfate on a synthetic medium such as B\(_{12}\) assay medium. At present, we cannot clearly understand the role of ammonium sulfate in the expression of the activity of the BGSs. It is considered that ammonium sulfate would support the expression of the activity of the BGSs either directly or indirectly.

Poch and Bezkorovainy\(^{23}\) have proposed a carrier for common metals, vitamins, or lipids, as one of the action mechanisms of \(\kappa\)-casein. On the other hand, Juillard \textit{et al.}\(^{28}\) investigated the specificity of milk peptide use by \textit{Lactococcus lactis}. They also found that the oligopeptide fraction in milk required some contaminating essential amino acids for the expression of the growth-stimulating activity. Thus, it has been observed that a peptide fraction has the activity only together with additional nitrogen sources. Generally, some nutrients such as peptides and sugars are incorporated into a cell through various accumulated energy forms, such as proton motive force and ATP-driven force.\(^{25}\) In our case, no cell growth was observed in B\(_{12}\) assay medium alone (Fig. 1). This result indicated that the intracellular metabolic flux would not be fully activated. As a result, intracellular ATP production and formation of a pH gradient between the inner and outer membranes did not occur. According to this hypothesis, the addition of ammonium sulfate, which is easily incorporated into cells, triggered the cell growth as an additional nitrogen source, then the BGSs were used by the metabolically activated cells. Oiki \textit{et al.}\(^{19}\) actually found the metabolic activation of cells in the presence of NRSP. If there were nitrogen sources in the medium enough to grow cells even slightly, the BGSs might be effectively used by the test strain. Although the studies on the uptake of sugars by \textit{Bifidobacterium} have been reported by several investigators,\(^{26-28}\) no publications on the peptide transport system are available. It is true that the BGSs would have higher activity owing to the peptide structure, but the extent of energy demand for the uptake of the peptides might characterize the limited bifidobacterial growth compared to other microorganisms. Furthermore, the peptide content in the medium used has been found to affect the regulation of proteolytic activity in \textit{Lactococcus lactis}.\(^{29}\) On the other hand, it has been reported that \textit{Bifidobacterium} uses peptides produced by the protease of lactic acid bacteria, which is used as a mixed starter in milk, due to its low proteolytic activity.\(^{30}\) From this viewpoint, the relationship between proteolytic activity of \textit{Bifidobacterium} and the content of nitrogen sources in a medium seems to be interesting. Thus, the presence of nitrogen sources in a medium could be significant in the use of peptides in these microorganisms.

At the industrial level, a high cell density cultivation of \textit{Bifidobacterium} can be done in media composed of expensive organic nutrients. As mentioned above, we could increase the activity of the BGSs, which alone showed no activity, by supplementing B\(_{12}\) assay medium with ammonium sulfate as a nitrogen source. \textit{Bifidobacterium} is known to be able to replace major nitrogen sources with ammonium salts.\(^{31}\) Actually, ammonium salts other than ammonium sulfate also could induce the expression of the activity of the BGSs (data not shown). Therefore, it is expected that a higher cell density culture should be brought by the combination of the BGSs derived from NRSP with cheap ammonium salts instead of expensive organic nitrogen sources.

\textbf{References}


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