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A Decapeptide, Growth Stimulator Identified from Natural Rubber Serum Powder for *Bifidobacterium bifidum*

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Natural rubber serum powder, which is by-product obtained in the production of latex rubber, has a strong growth-stimulating activity for *Bifidobacterium bifidum* JCM 1254. The retained fraction obtained by ultrafiltration (molecular weight cutoff 1000) showed a growth-stimulating activity in a dose-dependent manner on B_{12} assay medium with ammonium sulfate. One of the growth stimulators was purified from the retained fraction by acetone precipitation, solid-phase extraction with a hydrophobic pretreatment column, and multi-stage reversed-phase HPLC. An increase of 53-fold in the specific activity, and a recovery of 1.3% were obtained. The amino acid composition and N-terminal sequence analyses of this growth stimulator provided the structure of Ala-Thr-Pro-Glu-Lys-Glu-Glu-Pro-Thr-Ala. The molecular mass was 1075 by MALDI-TOF MS analysis. These results showed that this growth stimulator was a decapeptide with the sequence shown above. This is the first report that clarified the structure of an active peptide for the growth of *Bifidobacterium*.

INTRODUCTION

Bifidobacteria have been extensively studied on the basis of the very close correlation between their inhabitance in intestinal tract and promotion of the host health, recently referred to as a probiotic, which benefits the host by improving its intestinal microbial balance (Hentges, 1992), alongside of lactic acid bacteria. Compared with such clinical research, knowledges on biochemistry and physiology of *Bifidobacterium*, including carbohydrate and nitrogen metabolism, oxygen tolerance and synthesis of vitamins, seem to be less available. Of these works, search for growth stimulators characteristic to bifidobacteria, most of which have a nutritionally fastidious growth even in a full synthetic medium, is one of the most attractive topics in applied as well as fundamental aspects. Summarizing a recent review (Tamine *et al.*, 1995) and original reports, growth stimulators are generally classified into the following groups: (I) Bifidus factor 1; this growth factor consists of glycoproteins or enzymatic digests that contain sugars such as *N*-acetylglucosamine, glucosamine, and galactosamine, in milk and colostrums. (II)

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Bifidus factor 2; this represents non-glycosylated peptides derived from protein after hydrolysis using proteinase. However, none of them have been structurally identified. (III) Oligosaccharides; they act as energy or carbon sources for growth, and their chemical structures and outstanding selectivity for bifidobacteria have been sufficiently described. Therefore, these nondigestible nutritional compounds have been accepted as a prebiotic the availability of which has been also proved in *in vivo* model systems (Roberfroid, 1996; Fuller and Gibson, 1997). (IV) Other compounds that belong to none of the groups described above, and have been found from a variety of natural resources. Recently, 2-amino-3-carboxy-1,4-naphthoquinone was discovered in the cell-free filtrate and in the methanol-extract fraction of *Propionibacterium freudenreichii* that is used in the production of Swiss-type cheese (Mori *et al.*, 1997). The complete identification of this growth stimulator successfully enabled the action mechanisms to be studied at molecular levels. This fact led to the proposal of a new model for bifidobacterial growth stimulation by efficient turnover of an intracellular redox reaction via 2-amino-3-carboxy-1,4-naphthoquinone as an electron transfer mediator (Yamazaki *et al.*, 1998).

We have reported that natural rubber serum powder (NRSP), obtained in large quantities as by-product in the production of latex rubber, has a great potential as an additional candidate resource of growth stimulators for a wide range of bifidobacteria of human origin (Ishizaki, 1995). NRSP had the activity for *Bifidobacterium bifidum* in the same manner as casein enzymatic hydrolysates, both of which were able to grow the test strain on a medium deficient in nitrogen source. Moreover, NRSP markedly stimulated growth even in the nutritionally complete bifidobacterial medium (Oiki *et al.*, 1996). In our previous paper, we described a bioassay system, which is based on B_{12} assay medium with 0.25% ammonium sulfate, specifically to detect the activity of desired growth stimulators in NRSP, and obtained partially purified active fractions which all might be composed of many kinds of peptides (Etoh *et al.*, 1999). Nevertheless, although the significance of peptides for a sufficient growth of bifidobacteria in whatever synthetic medium or milk and related complex media has been well proved (Poch and Bezkorovainy, 1991; Gomes *et al.*, 1998; Gomes and Malcata, 1998), there are few reports attempting to clarify the function of peptides as a growth stimulator at molecular levels, which is in part due to the complicated nutrient requirement of bifidobacteria, especially with regard to nitrogen sources (Poch and Bezkorovainy, 1991). For the successful biochemical elucidation of the physiological response of *Bifidobacterium* to growth-stimulating peptides, accumulation of information on the chemical structures will be essential. In this paper, we report the purification of a growth-stimulating peptide from NRSP and the elucidation of its structure.

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 (Ishizaki, 1989).

Bioassay

The microorganism was grown in anaerobic jars (AnaeroPack Kenki, Mitsubishi Gas Chemical Co., Inc., Tokyo) in thioglycolate (TGC) medium without dextrose (Difco Laboratories, Detroit, MI, U.S.A.) and stored at 5 °C. The stored culture was first cultured in the bifidobacterium medium (Ishizaki, 1995) at 37 °C for 24 h. The culture broth was centrifuged at 8,000×g for 10 min. The precipitated cells were washed two times and suspended in a sterile solution of KCl (1.08%). The basal medium used for a bioassay was B_{12} assay medium (Difco) with 0.25% ammonium sulfate (Etoh *et al.*, 1999). 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 (A_{562}) after 40 h of cultivation. The growth experiments were done in duplicate or more each time, and resulting values were averaged in each experiment. In the purification of growth stimulator(s), the activity (A_{562}) of the fraction was defined as the difference in cell growth of *B. bifidum* observed after 40 h in B_{12} assay medium with 2.5 g/l ammonium sulfate, with and without the fraction. For preparation of a purification table, one unit (U) of activity of the growth stimulator was defined herein as the amount of the growth stimulator required for 1 mg increase in dry cell mass, which is converted from A_{562} by a standard curve, during 40 h of cultivation of the test strain in the assay system used. Protein concentration of each sample in the purification steps was calculated as a total amino acid content by amino acid composition analysis described below.

Purification of a growth stimulator from NRSP

NRSP was ultrafiltered with a membrane of molecular weight cutoff (MWCO) 1000 (Amicon, Inc., Beverly, MA, U. S. A.). The retained fraction was mixed with cold acetone the concentration of which was adjusted to 80%. The mixture was left at 4 °C overnight. The resulting precipitate was removed by centrifugation at 10,000×g for 10 min. The supernatant was dried by a Speed Vac Concentrator (Savant Instruments, Inc., NY, U. S. A.) and then dissolved in distilled water. The solution was put on a hydrophobic pretreatment column, tC_{18} Sep-Pak cartridge (Waters, Milford, MA, U. S. A.) and eluted successively with 3 ml of the solution of 0, 10, 20, 30, 40, and 50% acetonitrile in 0.05% trifluoroacetic acid (TFA). The fraction with the highest activity was dried and dissolved in distilled water as mentioned above. Further fractionation was done by reversed-phase HPLC as follows. In the first run, active components were eluted with a linear gradient of 0–40% acetonitrile in 0.05% TFA for 30 min through a column of TSKgel ODS-120A (Tosoh Co., Ltd., Tokyo: 25 cm×4.6 mm inner diameter) at a flow rate of 0.8 ml/min, while monitoring the absorbance at 210 nm. The fraction of the highest activity was used for the second run under these conditions except for a linear gradient of 0–15% acetonitrile for 60 min. The resulting active fractions were further analyzed by TSKgel ODS-80T_M column (Tosoh: 25 cm×4.6 mm inner diameter) with a linear gradient of

0–15% acetonitrile for 80 min. The purified sample was collected and dried with the Speed Vac Concentrator.

Mass spectrometry, amino acid composition and sequence analyses

The molecular mass of the purified growth stimulator was measured using a Voyager–RP MALDI–TOF spectrometer (Perspective Biosystems, MA, U. S. A.). In this analysis, an α–cyano–4–hydroxycinnamic acid solution was used as matrix. The amino acid analysis was done with a Hitachi L–8500 amino acid analyzer (Hitachi) after the purified sample was hydrolyzed in 6 N HCl under a vacuum at 110 °C for 24 h. The N–terminal amino acid sequence of the purified one was analyzed by Edman degradation with an automated gas–phase automatic sequence analyzer (PSQ–1, Shimadzu, Kyoto) with an on–line LC–6A HPLC system (Shimadzu).

RESULTS

Growth stimulation by the retained fraction of NRSP

Purification of growth stimulators from the ultrafiltrate (Etoh *et al.*, 1999), obtained

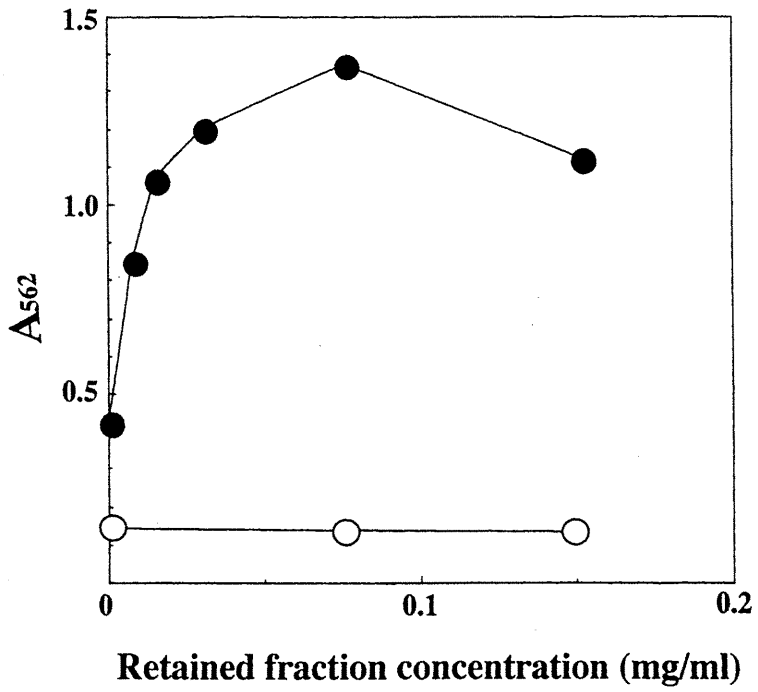

Fig. 1. Effects of concentrations of the retained fraction on *Bifidobacterium bifidum* JCM 1254. The retained fraction was obtained by ultrafiltration (MWCO 1000) of NRSP. B_{12} assay medium was mixed with the fraction in the presence of 2.5 g/l ammonium sulfate (●) and without ammonium sulfate (○).

by ultrafiltration (MWCO 1000) of NRSP, fell into a lot of difficulties because of interference by many kinds of amino acids and unidentified low-molecular-weight compounds. Therefore, we addressed the retained fraction for the isolation of growth stimulator(s). The retained fraction showed the activity only together with ammonium sulfate in a dose-dependent manner, like the peptide fraction from the ultrafiltrate (Etoh *et al.*, 1999), on B_{12} assay medium (Fig. 1). This finding suggested that the retained fraction would be composed of the substances with almost the same nature as the peptide fraction. However, the addition of the retained fraction above 0.1 mg/ml decreased the growth-stimulating activity.

Purification of a growth stimulator

Like the peptide fraction (Etoh *et al.*, 1999), dispersal over several active fractions and decrease in the activity were also observed in the process of the purification from the retained fraction. Poch and Bezkorovainy (1991) also encountered a similar phenomenon during the purification of growth stimulators from κ-casein digests. However, with the aim of identifying at least one active component, we concentrated fractions obtained in each purification step to a level capable of detecting a significant activity, and followed the fraction with the highest activity among all the active ones. First, the retained fraction was treated with 80% acetone to remove high molecular weight materials and to readily target active components with low molecular weight for simplification of the subsequent purification and identification. The specific activity of the supernatant was four-fold higher than that of the precipitate. The supernatant was put on a hydrophobic pretreatment column, Sep-Pak tC_{18}, and was fractionated by stepwise elution with several concentrations of acetonitrile. The fraction eluted with 10% acetonitrile had the highest activity of all the fractions eluted (data not shown), suggesting that relatively hydrophilic materials could be responsible for high growth-stimulating activity. This fraction was further fractionated by multi-stage reversed-phase HPLC. The chromatogram obtained by the first run with a TSKgel ODS-120A column is shown in Fig. 2a. The highest activity was observed in the fraction (F-15) eluted at approximately 15 min. F-15 was then put on the second run with the same column, where the slope of the gradient was more gentle than that in the first run (Fig. 2b). All the fractions with some activity were further analyzed by the third run, where the column was a TSKgel ODS-80T_M column, to confirm

Table 1. Purification of a Growth Stimulator from NRSP

Sample	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (-fold)
Retained fraction (MWCO 1000)	20	30.3	275	9.09	100	1
80% acetone supernatant	100	6.78	131	19.3	47.6	2.12
Sep-Pak tC_{18} eluate	10	2.30	163	70.7	59.3	7.78
Reversed-phase HPLC eluate (F-15d)	10	7.32×10^{-3}	3.52	481	1.28	52.9

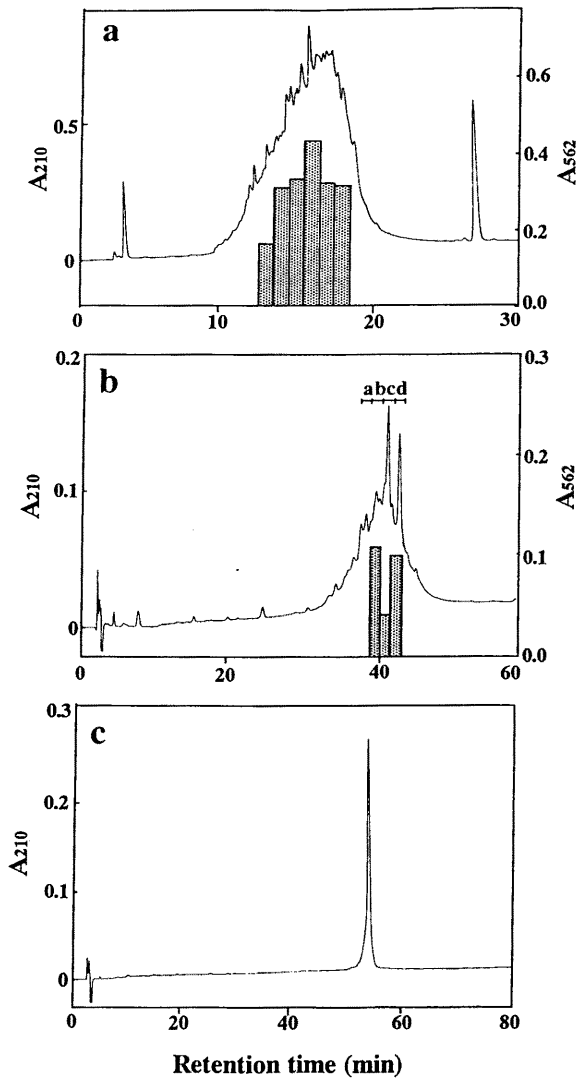

Fig. 2. Purification procedure of a growth stimulator from NRSP.
(a) The fraction eluted with 10% acetonitrile through the hydrophobic pretreatment column was chromatographed on a TSKgel ODS-120A column (first run). The fraction with the highest activity (F-15) was collected. (b) F-15 was chromatographed on a TSKgel ODS-120A column (second run). The eluates were divided into four fractions (F-15a-d). (c) F-15d was chromatographed on a TSKgel ODS-80T_M column to confirm its purity. Eluted fractions were monitored with the absorbance at 210 nm (left scale, solid line). A_{562} represents the extent of growth (right scale, shaded bars) in the assay medium supplemented with each fraction the protein concentration of which was 16 times that in the retained fraction (0.15 mg/ml).

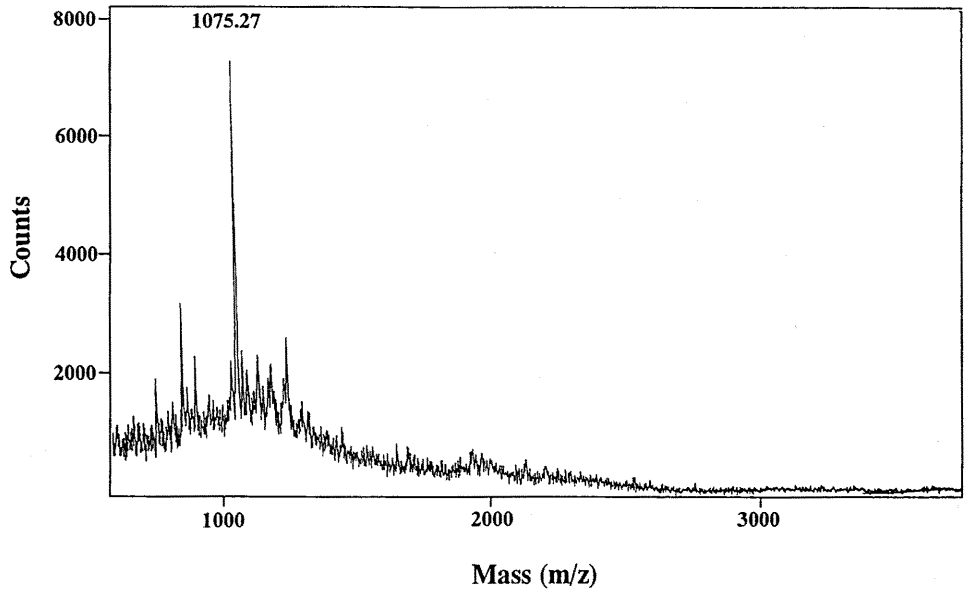

Fig. 3. MALDI-TOF mass spectrum of F-15d.

the purity of each fraction. As a result, the fraction (F-15d) was detected as a single peak with growth-stimulating activity (Fig. 2c), while the other unpurified fractions could not be resolved any more by using reversed-phase HPLC (data not shown). Apparent specific activity increased 53-fold and the recovery of the activity was 1.3% as shown in the purification table for F-15d (Table 1). It is not so surprising that the recovery of the activity was very low because growth-stimulating activity was present in the overall fractions separated in the process of the purification. Although the synergism of F-15d with other fractions was also expected on the growth stimulation, we used F-15d as a representative of the growth stimulators in the subsequent experiments.

Structure analysis of a growth stimulator

Since F-15d was positive in the ninhydrin reaction test, its amino acid composition was analyzed. The analysis gave the following composition: Glu(3), Lys(1), Ala(2), Thr(2), Pro(2). Furthermore, N-terminal sequence analysis clearly yielded the primary structure of Ala-Thr-Pro-Glu-Lys-Glu-Glu-Pro-Thr-Ala. MALDI-TOF MS analysis of F-15d showed that the molecular weight was 1075 (Fig. 3). This value was almost in agreement with that (1072) calculated from the amino acid sequence. Given these structural analyses of F-15d, one of the growth stimulators in NRSP was a decapeptide with a unique symmetric sequence at both terminals.

DISCUSSION

This is the first report that clarified the structure of an active peptide for the growth

of *Bifidobacterium*. The peptide was an unmodified decapeptide belonging to Bifidus factor 2 in the sense of a non-glycosylated peptide, unlike the crude glycomacropeptide fraction the sugar and polypeptide moieties of which were reported to be important for the activity of the bifidus factor by Azuma *et al.* (1984). Moreover, the peptide did not contain any sulfhydryl groups, chemical modification of which has proved to markedly decrease the growth-stimulating activity of κ-casein digests by Poch and Bezkorovainy (1991). This indicated that sulfhydryl groups would not be necessarily indispensable for growth-stimulating activity for *Bifidobacterium*. These contradictory findings on structure-function relationships suggest that bifidobacteria might possess a highly diverse and complex use manner even for a peptide.

Utilizable peptides for microorganisms exert several unique functions not only as nutritional sources but also as signal molecules, which are translationally synthesized and once secreted outside of the cell, in cellular functions and property such as sensing of population density (Rudner *et al.*, 1991; Koide and Hoch; 1994) and sporulation (Perego *et al.*, 1991) in *Bacillus subtilis*. Generally, it may as well be considered that exogenously derived peptides for bacteria would function as nutritional sources like the use of milk peptides for growth observed in *Lactococcus lactis* (Kunji *et al.*, 1996); they are irreversibly transported into the cell, followed by intracellular digestion by a variety of peptidases, and resulting amino acids are consequently used for protein synthesis or alternatively as a source of nitrogen or carbon. It is therefore unlikely that the isolated flawless peptide directly activates the expression of certain genes to stimulate the growth of the test strain. The presence of intracellular peptidases (Cheng and Nagasawa, 1985a, 1985b; Minagawa *et al.*, 1985; EL-Soda *et al.*, 1992) and extracellular proteinase(s) (Desjardins *et al.*, 1991) in *Bifidobacterium* could also support the possibility that the decapeptide would be responsible for growth stimulation as a nutritional source. Thus, bifidobacteria are likely to have an efficient peptide use system like *Lactococcus lactis* (Kunji *et al.*, 1996), some reports suggesting the significance of peptides rather than amino acids as a nitrogen source (Gomes *et al.*, 1998; Gomes and Malcata, 1998). More exactly mentioned, the peptide in this study would contribute to cell production as a growth promoter synergistically with ammonia, which is also effective as a nitrogen source in bifidobacteria (Matteuzzi *et al.*, 1978), derived from ammonium sulfate added to B_{12} assay medium (Etoh *et al.*, 1999).

The hydrophobicity and the molecular weight of peptide are always focused on discussing its efficiency of use by microorganisms. Such a specificity of peptides seems to strictly depend on the strains used. For example, Chen *et al.* (1987) demonstrated that hydrophilic casein digests were incorporated two-fold faster into the cell than hydrophobic ones in ruminal bacteria, by evaluating the growth rate or the metabolite production. On the other hand, Juillard *et al.* (1998) concluded that basic and hydrophobic peptides with molecular mass ranging between approximately 600 and 1,100 Da were most preferentially used by *Lactococcus lactis*, by monitoring disappearance of peptides in the medium by HPLC although this description only shows the facility in the transport of peptides by the cells. In our case, the peptide, which was obtained as a consequence of screening for higher active growth stimulators during the purification, was highly hydrophilic and with a length of ten amino acid residues. Although the definition itself of 'preferential utilization' thus appears to be somewhat different in each case, our results

reflect at least how efficiently this peptide was incorporated into the overall system to produce cell mass.

Another possible action mechanism of the peptide is never ruled out, while the above description is a plausible hypothesis. Poch and Bezkorovainy (1991) have supposed that cysteine-containing peptide may be required as membrane carriers of nutrients, although this idea has not been sufficiently proved. On the other hand, Zhao *et al.* (1996) have suggested that a growth-stimulating peptide isolated from bovine hemoglobin may target the periplasmic binding-protein for the uptake of nutrients to activate the corresponding transport systems of Gram-negative bacteria. Recently, lactoferrin, an iron binding-glycoprotein found abundantly in human milk, was reported to have a growth-stimulating activity for *Bifidobacterium breve* by transferring iron directly to the cell (Catchpole *et al.*, 1997). Thus, our peptide also may be associated with the acceleration of the uptake of some nutrients rather than with anabolism for cell production. Phytochelatin has been known to be a heavy metal-entrapping polypeptide, the biosynthesis of which occurs under the stress conditions caused by heavy metals, resulting in the expression of tolerance against such toxic metals for higher plants (Grill *et al.*, 1985). This polypeptide stably entraps metals with the repeated sulfhydryl groups, which coordinate to the metals in a multidentate way, in the sequence polymerized with γ-Glu-Cys (Grill *et al.*, 1985). Interestingly, a unique inverted repeat sequence was also clearly observed at both terminals in the purified peptide here. As far as the isolated peptide is concerned, the structural feature led us to suppose that this peptide might be a membrane carrier for particular metals required for growth stimulation. Furthermore, it is noteworthy that both peptides arise from higher plants.

It is greatly significant that a structurally identified peptide was obtained as a growth stimulator for *Bifidobacterium*. Using the synthetic derivatives based on the sequence obtained as a bioprobe, we could not only elucidate the function of the peptide but also dramatically develop new avenues for the related biochemistry and physiology of *Bifidobacterium*, which have not been profoundly investigated due to the difficulty of approaches to this strain.

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