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Bifidus Factors in Carrot. I. Purification and Some Properties[1]

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Procedures for the purification of acidic growth factors for *Bifidobacterium bifidum* from carrot were developed. These involved adequate solvent extraction and subsequent successive chromatography on Amberlite IR-45, IRC-50, IRA-68, and SE, DEAE, QAE Sephadex which included a proper precipitation procedure for the SE-Sephadex eluate, and preparative paper electrophoresis.

Among five kinds of growth factors separated, the major growth factor and the most acidic factor were highly purified. Both factors differed from coenzyme A and its known precursors, while one of the weaker acidic factors coincided with D-pantetheine 4′-phosphate in chromatographic and electrophoretic behaviors.

There are many kinds of microorganisms in human intestines. It is well known that they play some important roles in maintaining human health.

Statistical investigation showed that the mortality and morbidity of bottle-fed infants were greater than those of breast-fed infants, the fact coincided with a difference of intestinal bacterial flora. In the case of breast-fed infants, *Bifidobacterium bifidum* was prevalent in their intestines, while gram-negative bacilli such as *E. coli* were rather prosperous in case of bottle-fed infants.[3] This suggested that *B. bifidum* might have some positive functions in maintaining good health of milk-fed infants. Following these observations many investigators have tried to make *B. bifidum* grow artificially *in vivo* and *in vitro*.

B. bifidum (previously designated as *Lactobacillus bifidus*), a strictly anaerobic bacteria which produces lactic and acetic acids,[4] keeps the intestinal tract acidic so that it will be protected from propagation of other injurious bacteria. *Bifidobacterium* is classified into many strains on the basis of morphological types, fermentation patterns of sugars, and antigenic characteristics.[5]

In accordance with varieties of the strains, several kinds of growth promoting substances for *B. bifidum* are known. Lactulose[6] derived from lactose was first named as a bifidus factor (Petuely factor). This increased *B. bifidum in vivo* but not *in vitro*. Oligosaccharides containing N-acetylglucosamine[7] purified from human milk and hog gastric mucin were named together as bifidus factor I (*var. penn.* factor), which were only effective on a variant, *B. bifidum* var. *pennsylvanicus*, and not on other regular strains. Some peptides obtained from enzymatic digest of casein[8] had effects on a strain of Tissier and were named as bifidus factor II (Raynaud factor). There were other peptide-like substances such as Strepogenin

1) This work was partly presented at the 89th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, April 1969.
2) Location: *Hongo, Bunkyo-ku, Tokyo.*
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7) P. György and C.S. Rose, *Proc. Soc. Exp. Biol. Med.*, **90**, 219 (1955).
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factor[9] and Supplementary factor,[10,11] but it has not been confirmed whether they were similar to Raynaud factor. However these factors were only effective on limited strains with rather small populations, and were unable to support growth of other commonly distributed strains of *B. bifidum*.

In the field of pediatrics, on the other hand, vegetable soups, especially carrot soup, have been used for a long time in the treatment of infant diarrhea and undernourishment.[12] The mechanism of action of carrot has been studied from the standpoints of cinderlike substance therapy[13] and growth of *B. bifidum*. Kuromiya first showed that the majority of strains of *B. bifidum* except var. *pennsylvanicus* grew well in the presence of carrot extract.[14] This suggested the existence of widely effective bifidus factors in carrot. Based on the information, Kanao, *et al.* attempted to purify an active substance from a hundred kilograms of carrot powder, and obtained 1.7 miligrams of forty thousand times purified and still impure substance which was a kind of vitamin with strong acidic character.[15] Tamura, *et al.* suggested that the active substance was identical with D-pantetheine 4′-phosphate (P-PaSH) or a closely related compound.[16] This conclusion coincided with the finding of Rose, *et al.* who had demonstrated the growth promoting activity of D-pantethine (PaSS) (*Lactobacillus bulgaricus* factor[17]) on var. *pennsylvanicus* and other strains of *B. bifidum*.[10] Yoshioka, *et al.* examined the growth activities of coenzyme A, its precursors,[18] and carrot extract for about fifty strains of *B. bifidum* isolated from the stools of breast–, bottle– and mixed–fed infants, and found that the activities of coenzyme A and its precursors varied for each of the strains, while carrot extract was active for most of the strains. This indicates the presence of some factors other than coenzyme A or its known precursors in carrot extract.[19]

Again we attempted to purify bifidus factors from roots of *Daucus carrota* L.

Experimental

All evaporations of sample solutions were carried out *in vacuo* below 40°.

Materials——Carrot (*Daucus carrota* L.) roots (1000 kg) were cut crosswise, boiled for 5 minutes, dried on a hot roller with crushing at 115° for 1.5—2 minutes, and reduced to a powder (100 kg).

The ion exchangers used in this study and their sources are: Amberlite IR-45 anion exchanger (capacity 1.9 meq/ml), Amberlite IRC-50 cation exchanger (capacity 3.5 meq/ml), Amberlite IRA-68 anion exchanger (capacity 1.6 meq/ml) from Rohm & Haas Co., and SE-Sephadex C-25 cation exchanger (capacity 2.3 ± 0.3 meq/g), DEAE-Sephadex A-25 anion exchanger (capacity 3.5 ± 0.5 meq/g), QAE-Sephadex A-25 anion exchanger (capacity 3.0 ± 0.4 meq/g) from Pharmacia Fine Chemicals Co.

Toyo Roshi No. 51A and Whatman No. 3MM Filter Paper were used for paper electrophoresis.

D-Pantethine (PaSS), calcium D-pantetheine 4′-phosphate·$5H_2O$ (P-PaSH), calcium D-pantethine 4′,4″-diphosphate·$4H_2O$ (P-PaSS) and coenzyme A·3Li (CoA) were kindly provided by the Research Laboratories, Daiichi Seiyaku Co., Ltd. All other chemicals used were commercially available guaranteed reagents.

Apparatuses——Large scale procedures were carried out with plants in Daiichi Seiyaku Co., Ltd. Stainless or glass-lined tanks were used. Cylinders for column chromatography and all tubings were made of polyethylen.

9) D.W. Wooley, *J. Biol. Chem.*, **172**, 71 (1948); H. Gyllenberg, M. Rossander and P. Roine, *J. Gen. Microbiol.*, **9**, 190 (1953).
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14) M. Kuromiya, *Ochanomizu Med. J.*, **8**, 88 (1960) (in Japanese).
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A concentrated aqueous solution with a circulating steam evaporator was finally evaporated to dryness with rotary evaporator through a trap cooled by methanol–dry ice.

Amino acid composition was determined by Yanagimoto Model LC2 Amino Acid Analyzer. Toyo Roshi Co. Type C was used for paper electrophoresis.

Preparation of Sample for Microbiological Assay——An aliquot from every fraction was evaporated to dryness and, when the residue contained a larger amount of ammonium carbonate or ammonium bicarbonate which inhibited the growth of *B. bifidum*, the salts were removed by keeping the residue in a desicator with both sulfuric acid and sodium hydroxide under reduced pressure for a day or two at 37.5°.

Microbiological Assay Procedure——*B. bifidum* N4, one of the common strains in feces of infants isolated by Negishi[20] was used throughout these studies which demonstrated a specific and stable dose–response curve with the unknown bifidus growth factors in the aqueous extract of carrot.

The strain N4 was preincubated with 2 ml of the stock medium[19] in an atmosphere of N_2–CO_2 (9:1 v/v) at 37.5° for 24 hours, centrifuged and washed two times with the basal medium[19] which modified the György's[21] by replacing the enzymatic digest of casein with casamino acids (acid-hydrolyzed casein) and omitting calciumD-pantothenate. The strain was then suspended in 12 ml of the basal medium supplemented with 0.5 g of ascorbic acid, and the suspension was used as an inoculum.

The unit was defined as the activity which 1 mg of 80% methanol extract of carrot powder brought about.

Aliquots (equivalent to about 100 units) of sample were dissolved in 10.5 ml of the basal medium, and the solution was divided into 0.2, 0.4, 0.6, 0.8, 1.0, and 2.0 ml and added with the basal medium of 1.8, 1.6, 1,4, 1.2, 1.0, and 0 ml respectively to make final volumes 2 ml. The same treatment was done every time with a standard carrot extract (100 units). After autoclaving them at 115° for 5 minutes and cooling, one drop of the inoculum was added to each of them. They were incubated in an atmosphere of N_2–CO_2 (9:1 v/v) at 37.5° for 48 hours. Growth of the organisms was estimated by titrimetric assay (0.1N NaOH) of the acid produced, adding each 0.1 ml of the ethanol solution of bromothymol blue (0.1%) and neutral red (0.05%) as an indicator to the medium. The activity was calculated from a calibration curve with standard carrot extract.

Bioautography[22]——The twice washed N4 strain with the sterilized basal medium as described above was suspended in the medium to get optical density of 0.3 by the aid of Coleman Jr. Spectrophotometer at 650 mμ. This suspension (11 ml) added with 0.1 g of ascorbic acid was used as an inoculum.

Eighty ml of the basal medium with 1.5% of agar (Bacto-Agar, Difco) was autoclaved for 15 minutes at 115°, and after cooling to 40°, 2.4 ml of the inoculum was added. A uniform sheet of medium (about 4 mm thick) was immediately prepared by pouring the molten seeded agar onto a glass plate (10 × 20 cm) which was sterilized by ultraviolet (UV) light irradiation and set in a tray with a little larger space. After the seeded agar was solidified, it was taken out of the tray together with the glass plate, and a chromatogram or an electrophorogram was placed on its surface. This setting wrapped with Krewrap (Kureha Chemical Industry Co., Tokyo) was incubated for 16 hours at 37.5° under an anaerobic condition of N_2–CO_2 (9:1 v/v). Detection was done with growth zones.

Result

Purification in a Large Scale

After a satisfactory purification procedure of bifidus factors had been established in each step, the scale of process was enlarged for 100 kg of the dried powder of carrot as shown in Chart 1.

Step 1. Extraction from Carrot Powder——Water and methanol had been tested as solvents for extraction of active substances from dried carrot powder because of their insolubility to organic solvents.[15] However, when water was used, emulsification occurred which prevented the separation of the solution from insoluble sediment. Water extraction had other faults such as large quantities of extractable impurities and growth of mold in a time-consuming operation of a large scale procedure. In the case of methanol extraction, the efficiency decreased and much quantity of methanol under reflux was required for a good recovery of activity. After testing simplicity of extraction procedure, weight of extract and recovery of activity, a mixture of water and methanol (1 : 4) was selected as the best solvent

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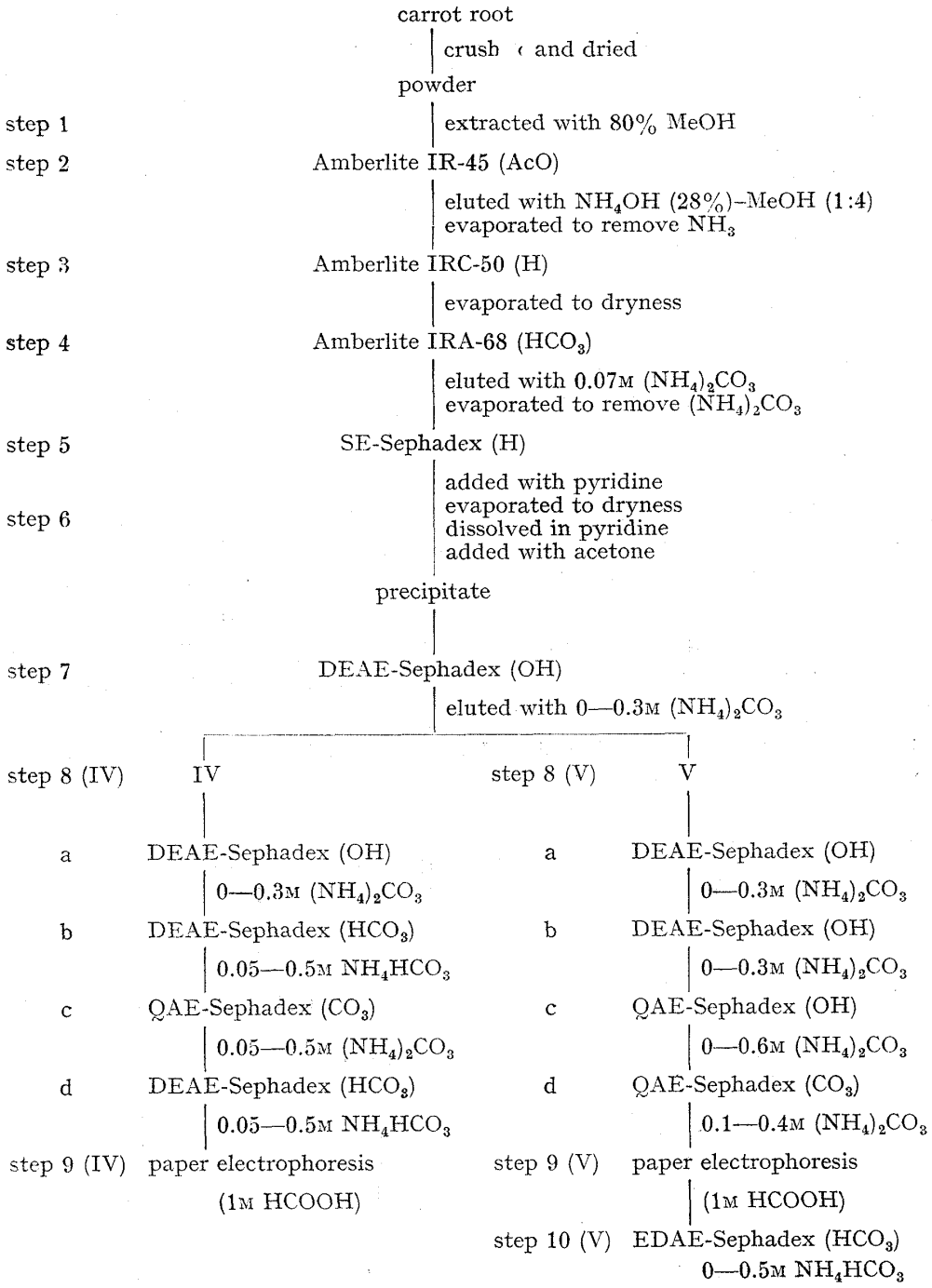

Chart 1. Isolation and Purification of Two Novel Factors from Carrot Root

(Table I). With the solvent, the active substances were effectively extracted merely by eluting a column of the dried carrot powder.

Large Scale Procedure: Every 25 kg of the powder was put in a stainless tank (600 liters capacity), and a mixture of methanol, water, and acetic acid (4 : 1 : 0.005, v/v) (250 liters) was added. After stirring for 1 hr at room temperature, the solution was filtered off the insoluble sediment through a stainless Buchner funnel (80 cm diameter).

The sediment was furthre treated twice with every 250 liters of the same solvent. The last 250 liters of filtrate was used for the first extraction of the next 25 kg of the powder. The addition of acetic acid was purposed for a direct application of the filtrate to step 2. The specific activity of the extract was assumed ot be 1 unit per mg as indicated in Experimental. The results are summarized in Table V.

Table I. Activities and Weights extracted from Carrot Powder[a] by Various Mixtures of Water and Methanol

Solvent system H_2O–MeOH (v/v)	Weight extracted (g)	Activity extracted (ratio)
5:0	6.8	1.0[b]
4:1	6.7	1.2
3:2	6.3	1.2
2:3	6.1	1.0
1:4	4.6	1.6
0:5	4.6	1.0

a) Ten grams of carrot powder suspended in 100 milliliters of each solvent system were stirred for 1 hour at room temperature. The solution was filtered off, and the residue was treated similarly twice with the same solvent. The combined solution was evaporated to dryness.
b) The total activity extracted with water was assumed to be 1.0.

Step 2. Preliminary Separation of Activities with Amberlite IR-45——An anion exchanger, Amberlite IR-45, was fit for adsorbing substances of the strong acidic character like P-PaSH and was available for the organic of 80 % methanol without any apparent decrease of the exchange capacity. Acetate form of the exchanger gave a good recovery of the activity, although the free form caused a marked decrease of the recovery (see discussion). The filtrate in step 1 was applied to an Amberlite IR-45 acetate column, and after washing the column with 80 % methanol containing 0.1% acetic acid (fraction of neutral and basic substances), the activities were eluted with methanol and 28% ammonia water (4 : 1) (fraction of acidic substances). The results are shown in Table II.

Table II. The Effect of Weak Anion Exchangers, Amberlite IRA-93 and IR-45, on Recovery of Weight and Activity

	Recovery	
	Weight (%)	Activity (%)
80% MeOH extract	100[a]	100[a]
IR-45 (AcO) A[b]	92	75
B[b]	8	80
A+B	100	155
IRA-93 (OH) A	90	0
B	10	7
A+B	100	0
A+P-PaSH or PaSS[c]	—	0
IRA-93 (AcO) A	86	42
B	14	45
A+B	100	87
A+PaSS[c]	—	109

a) Both weight and activity of 80% methanol extract were assumed to be 100%.
b) The extract (25 g) in 80% methanol (540 ml) from step 1 was applied on a column packed with each anion exchanger (80 ml), and the column was washed with 80% methanol (400 ml). The combined solution was evaporated to dryness, and the fraction containing neutral and basic substances was designated as A. Then the column was eluted with methanol-28% ammonia water (4:1) (300 ml)and the effluent was evaporated to dryness, designated as B, which was a fraction containing acidic substances.
c) An amount of PaSS or P-PaSS corresponding to 50% activity was added.

Large Scale Procedure: Every 750 liters of the filtrate from step 1 was passed through Amberlite IR-45 acetate column (60 liters) about 4 hr ,and the column was washed with the same solvent as in step 1 (200 liters). Both effluents were combined. The column was then

eluted with a mixture of methanol and 28% ammonia water (4 : 1) (150 liters) over an hour. The ammoniacal effluent was treated according to the following step. The results inferred from a sampling were summarized in Table V.

Step 3. Removal of Ammonium Ion with Amberlite IRC-50——In order to remove ammonium ion from ammonium acetate formed in step 2, strong acidic ion exchanger like Amberlite IR-120 was not fit for this purpose because the strong acidity of the effluent resulted in decomposition of active substances during concentration. A satisfactory recovery of activity was obtained through Amberlite IR-50 (H) with 80% methanol elution.

Large Scale Procedure : One-third of the ammoniacal effluent from step 2 was concentrated to a small volume to remove excess of ammonia. The concentrated water solution (20 liters) mixed again with methanol (80 liters) was passed through an Amberlite IRC-50 (H) column (65 liters) over 1.5 hr, and the column was washed with 80% methanol (200 liters). The combined solution was evaporated to dryness. Another two-thirds was similarly treated after conditioning the resin. The results are summarized in Table V.

Step 4. Rough Separation of Activities with Amberlite IRA-68——This step was taken to make a rough separation of active substances and remove larger amounts of colored, mud-like sediment. The dried sample from step 3 dissolved in water was equilibrated in a flask with Amberlite IRA-68 (HCO_3) and after pH value of the mixture became 6, the lightly-colored solution together with the heavily-colored resin was applied to a column of the same ion exchanger. The completed column was washed with water saturated with carbon dioxide and then eluted with a linear gradient of ammonium carbonate from 0 to 0.1 M. Fractions were monitored for the growth activity of *B. bifidum*. The elution diagram indicated the existence of fast- (peak 1) and slow-moving (peak 2) active substances, and the behavior of the latter was similar to P-PaSH (Fig. 1a). A method of stepwise elution which would lend itself to a large scale procedure was also tested, and accepted to be efficient to separate peak 1 and 2 (Fig. 1b). A gradient elution pattern by 2% ammonia water instead of 0.1 M ammonium carbonate showed only one peak of activity with almost 100% yield (Fig. 1c). The results of stepwise elution are shown in Table III.

Large Scale Procedure: After conditioning Amberlite IRA-68 (OH) (30 liters) to bicarbonate form with dry ice (10 kg), 20 liters of the resin was packed in a cylinder. The remaining 10 liters of the resin was added to a half of the dried materials from step 3 (about 2 kg)

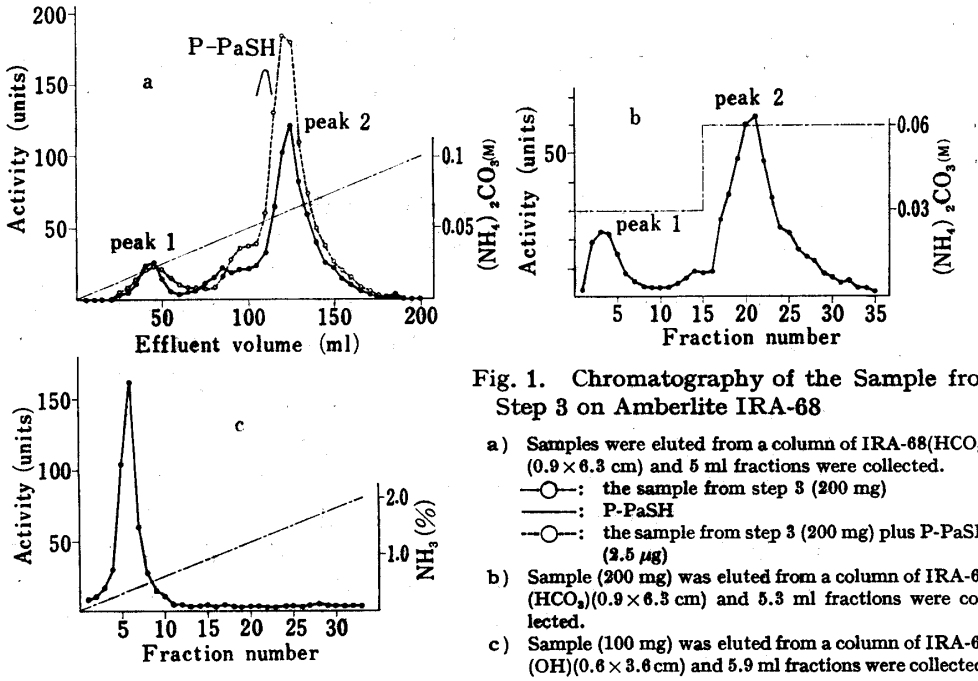

Fig. 1. Chromatography of the Sample from Step 3 on Amberlite IRA-68

a) Samples were eluted from a column of IRA-68(HCO_3) (0.9×6.3 cm) and 5 ml fractions were collected.
—○—: the sample from step 3 (200 mg)
———: P-PaSH
--○--: the sample from step 3 (200 mg) plus P-PaSH (2.5 μg)

b) Sample (200 mg) was eluted from a column of IRA-68 (HCO_3)(0.9×6.3 cm) and 5.3 ml fractions were collected.

c) Sample (100 mg) was eluted from a column of IRA-68 (OH)(0.6×3.6 cm) and 5.9 ml fractions were collected.

Table III. Stepwise Elution of the Sample from Step 3 (1.0 g, about 10000 Units) on a Column of Amberlite IRA-68 (HCO_3, 15 ml)

Eluate	(ml)	Weight (mg)	Total activity (units)	Specific activity (units/mg)
H_2O satd. with CO_2	(100)	229	945	4
0.03M $(NH_4)_2CO_3$	(200)	307	1040	3
0.07M $(NH_4)_2CO_3$	(400)	417	4550	11
2% NH_4OH	(300)	69	inhibited	—

dissolved in 10 liters of water. After equilibration completed (pH 6), both solution and resin were quantitatively applied to the arranged column. The completed column was first washed with water saturated with carbon dioxide and eluted with 300 liters of ammonium carbonate (0.03M), 600 liters of ammonium carbonate (0.07M), and 150 liters of ammonia water (2%) successively. Each effluent was evaporated to dryness. Ammonium carbonate was completely removed during the concentration.

The sample from 0.07M ammonium carbonate effluent with appreciable amounts of crystalline substances was further purified according to the following steps. The results are summarized in Tavle V.

Step 5. Change of Ammonium- to Pyridinium Salts with SE-Sephadex——Since the crystalline substances from step 4 had no growth promoting activity for *B. bifidum*, removing these crystalline substances would result in a further increase of specific activity in peak 2. For the purpose, it was desirable to use a proper organic solvent which dissolves most of the crystalline substances, but dose not dissolve the growth factors. However, no organic solvents like pyridine, acetone, and benzene dissolved the crystals because of their being ammonium salts, so the sample from step 4 was subjected to a SE-Sephades (H) column to change all ammonium salts in it to free acids. The column was washed with water until the pH of the eluate became neutral. This treatment served not only for changing them to free acids but for adsorbing colored impurities and a trace of amino acids. The direct drying of the effluent even *in vacuo* caused a decrease of total activity (about 70% recovery) and of specific activity owing to its low pH value of 1.8. But almost 100% recovery of total activity was obtained when the effluent was similarly evaporated to dryness at pH 5.0 after addition of pyridine, in this case the pyridinium salts solidified as paste instead of crystals.

Large Scale Procedure: The dried sample from 0.07M ammonium carbonate elution of step 4 (2.8 kg) dissolved in 40 liters of water was passed through a SE-Sephadex (H) column (15 kg), and the column was washed with 385 liters of water. The effluent (pH 1.8) was evaporated to dryness at pH 5 after addition of pyridine (1.5 liters).

Step 6. Precipitation with Acetone from the Pyridine Solution——The paste (pyridinium salts) from step 5 was soluble in pyridine but insoluble in acetone or benzene. Therefore, the adequate combination of pyridine and acetone was taken. After some preliminary tests, three volumes of acetone were added to one volume of pyridine solution of the paste, and the resulting precipitate was collected in which total activity was recovered in 70% yield, and the specific activity increased about ten-fold. The results are shown in Table IV. Most of the crystalline substances removed from the solution were identified with L-malic acid comparing its physical and chemical properties with the authentic sample.

Large Scale Procedure: The paste from step 5 (3.0 kg) was dissolved in 30 liters of pyridine by slightly warming, and 90 liters of acetone was added to the solution. The resulting precipitate sometimes obtained as an oily subtsance was collected by decantation and/or with Buchner funnel and washed with 4 liters of the same mixed solution of pyridine and acetone. The recovery of activity was 67% as expected, but the specific activity was only about half of the value obtained in the small scale.

Table IV. Treatment of the Pyridinium Paste from Step 5[a)]

	Weight (mg)	Total activity (units)	Specific activity (units/mg)
Supernatant	84.3	290	3
Precipitates	5.7	590	104

a) The pyridinium paste (90 mg, about 900 units) from step 5 was dissolved in 1 ml of pyridine, and 3 ml of acetone was added to the solution. The precipitates produced were separated from the supernatant by centrifugation.

Step 7. Further Separation of the Growth Factors with DEAE-Sephadex——The precipitate from step 6 dissolved in water was applied to a DEAE-Sephadex (OH) column, which was washed with water and eluted with a linear gradient of ammonium carbonate from 0 to 0.3M. The active substances were separated in five peaks at concentrations of ammonium carbonate of 0M (I), 0.04M (II), 0.09M (III), 0.12M (IV) and 0.21M (V) as shown in Fig. 2.

The retention volumes of peaks I, II, III coincided with those of PaSS, P-PaSH and P-PaSS respectively, while there were no substances known corresponding to the major peak IV and the following peak V. Therefore the further purification was carried on with these peaks as described in the following steps.

Large Scale Procedure: The precipitate from step 6 (225 g) dissolved in 4.5 liters of water was passed through a DEAE-Sephadex (OH) column (22.5 liters) which was washed with 105 liters of water (I), and then the linear gradient elution was performed with 315 liters of water in the mixing chamber and 315 lietrs of 0.21M ammonium carbonate in the reservor flask. The effluent was separated in three fractions according to the concentrations of eluate, *i.e.* 0—0.08M (II), 0.08—0.12M (III) and 0.12—0.21M (IV). The column was finally eluted with 0.3M ammonium carbonate (120 liters) (V). Each fraction was evaporated to dryness.

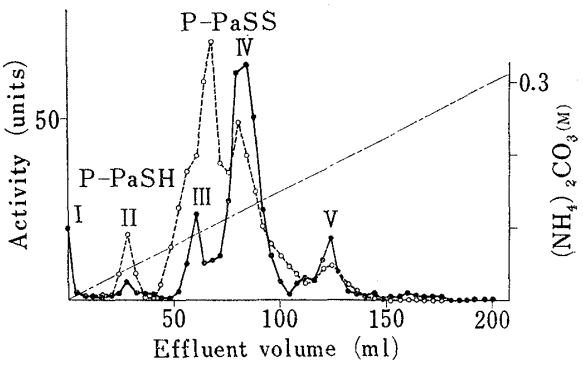

Fig. 2. Gradient Elution of the Sample from Step 6 from a Column of DEAE-Sephadex (OH) (0.8×5.0 cm)

4 ml fractions were collected.
—●—: the sample from step 6 (25 mg)
---○---: the sample from step 6 (12.5 mg) plus P-PaSS (5 μg) containing a small amount of P-PaSH

Recoveries of weight and activity in the steps of large scale purification described above are summarized in Table V.

Table V. Purification of Bifidus Factors by the Large-Scale Method

Purification step	Weight (g)	Total activity (units)	Specific activity (units/mg)	Yield of activity (%)
1. 80% MeOH extract	55000	5.5×10^7	1	100
2. IR-45 (AcO) ammonia effluent	4400[a)]	4.4×10^7	10	80
3. IRC-50 (H)	4500	4.2×10^7	9	76
4. IRA-68 (HCO_3) 0.07M $(NH_4)_2CO_3$	2800	2.0×10^7	7	36
5. SE-Sephadex (H)	3000	1.8×10^7	6	33
6. Pyridine–acetone (1:3) precipitates	230	1.2×10^7	52	22
7. DEAE-Sephadex (OH) I	50	0	0	0
II	145	1.2×10^6	8	2
III	8.5	8.5×10^5	100	2
IV	10.8	3.1×10^6	290	6
V	4.0	6.0×10^5	150	1

a) Inferred from the data of sampling.

Isolation of a Bifidus Factor from Fraction IV

Step 8 (IV). Chromatography with Anion Exchange Sephadex——a) Fraction IV (10.8 g) from step 7 dissolved in 108 ml of water was applied to a DEAE-Sephadex (OH) column (1.08 liters), which was washed wit 4.5 liters of water and eluted with a linear gradients of ammonium carbonate from 0 to 0.3M (86 liters). Fraction of 2 liters each were collected. Activity was observed in fractions from No. 16 to No. 40 which were collected and evaporated to dryness (3.93 g, 647 units/mg, 2.54×10^6 units).

b) The collected sample (3.93 g) dissolved in 800 ml of 0.05M ammonium bicarbonate was applied again to a DEAE-Sephadex (HCO_3) column (500 ml) preliminary equilibrated with 0.05M ammonium bicarbonate, and washed with 1.4 liters of 0.05M ammonium bicarbonate. The bifidus factor was eluted with a 0.05 to 0.5M ammonium bicarbonate linear gradient starting with 10 liters of each solution. Fractions of 1 liter each were collected, and fractions No. 8 to No. 12 were collected and evaporated to dryness (1.727 g, 1290 units/mg, 2.23×10^6 units).

c) The sample (1.727 g) dissolved in 200 ml of 0.05M ammonium carbonate was then applied to a QAE-Sephadex (CO_3) column (200 ml) equilibrated with 0.05M ammonium carbonate, and washed with 700 ml of the same solution. The factor was eluted with a 0.05M to 0.5M ammonium carbonate linear gradient starting with 4 liters of each solution. Fractions of 200 ml each were collected, and No. 15—No. 17 were evaporated to dryness (Fig. 3a, 0.53 g, 4180 units/mg, 2.21×10^6 units).

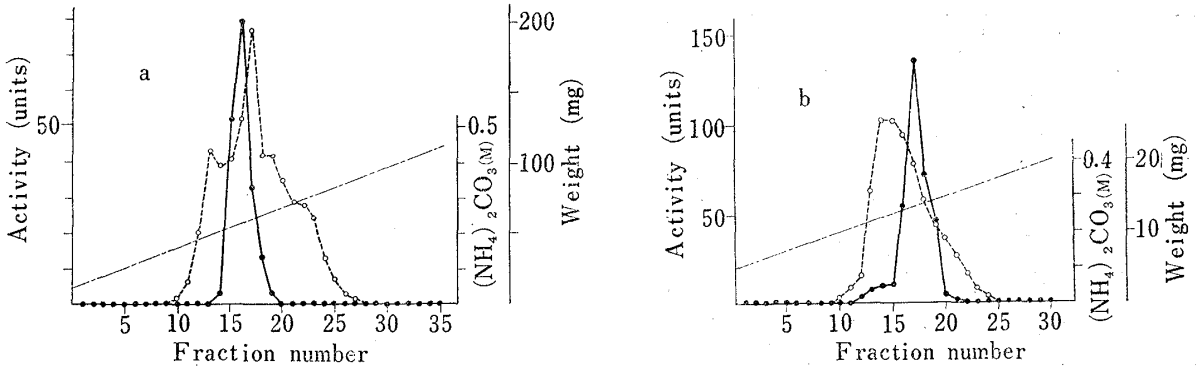

Fig. 3. Chromatography of IV (a) and V (b) on QAE-Sephadex

—●—: activity —○—: weight

d) The sample (0.53 g) was further purified by chromatography similar to step b). Dissolved in 40 ml of 0.05M ammonium bicarbonate, the sample was placed on a DEAE-Sephadex bicarbonate (40 ml) which was washed with 250 ml of 0.05M ammonium bicarbonate and then eluted with a linear gradient of ammonium bicarbonate from 0.05 to 0.5M (2 liters). Fractions of 50 ml each were collcted, and No. 11—No. 13 were evaporated to dryness (163 mg, 12000 units/mg, 1.95×10^6 units).

Table VI. Migrated Distances (cm)[a] of Factors from IV and V, and P-PaSH against Anode on a Filter Paper after 2 Hours at 11.8 v/cm

Sample	pH 2.0	pH 4.2	pH 8.0
Factor from IV (200000 units/mg)	7.0	6.5	7.5
Factor from V (310000 units/mg)	5.7	5.5	5.6
P-PaSH	4.5	4.8	5.0

a) corrected with glucose.

Step 9 (IV). Preparative Paper Electrophoresis——Paper electrophotograms obtained with 0.01 M ammonium bicarbonate (pH 8.0), acetic acid–pyridine–water (2:1:47) (pH 4.2) and 1 M formic acid (pH 2.0) showed that the factor had a larger mobility against anode than P-PaSH or CoA (Table VI). Particularly with 1 M formic acid, a good separation of the factor from P-PaSH or colored and fluorescent impurities was achieved as shown in Fig. 4.

The following preparative paper electrophoresis was conducted with 1 M formic acid: Aliquots (10 mg) from Step 8 (IV)-d spotted in a line (10 cm) on 10 % formic acid-washed Whatman No. 3 MM filter paper (14 cm in width) were subjected to electrophoresis at 5.9 volts per cm for 5 hr. After drying at room temperature, a strip (1 mm in width) along the current was cut from the paper electrophorogram around center, and the strip was used to detect the factor by bioautography. Then, the most active part was cut from the paper, eluted with water, and evaporated to dryness. The same treatments were repeated at least four times to get the 200000-fold purified factor which was changed to a calcium salt through a SE-Sephadex column (Ca) (3 mg, 200000 units/mg, 6×10^5 units).

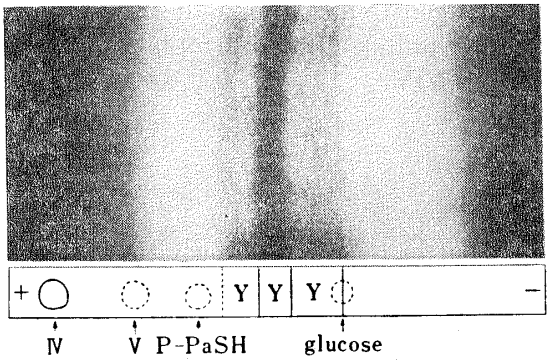

Fig. 4. Paper Electrophorogram of the Sample from Step 8(IV)-d

Y designates yellow band. The white bands on the photograph taken under a UV-lamp (365 mμ) demonstrate some fluorescent substances. Activities were detected by bioautography, and IV and V designate the factor in fractions IV and V respectively. The pattern of impurities in fraction V is almost the same as in fraction IV.

Further Purification of a Bifidus Factor in Fraction V

Fraction V (6.0 g) from Step 7 contained much insoluble materials. The aqueous solution of fraction V was centrifuged and the precipitate was washed several times. All the supernatants were evaporated to dryness (4.0 g, 150 units/mg, 6.00×10^5 units).

Step 8 (V). Chromatography with Anion Exchange Sephadex——a) The supernatant (4.0 g) dissolved in 40 ml of water was applied to a DEAE-Sephadex (OH) column (400 ml), which was washed with 1.6 liters of water and then eluted with a linear gradient of ammonium carbonate from 0 to 0.25 M (26 liters). Fractions of 640 ml each were collected, and No. 23—No. 36 were evaporated to dryness (850 mg, 350 units/mg, 2.98×10^5 units).

b) The sample (850 mg) dissolved in 8 ml of water was applied to a DEAE-Sephadex (OH) column (80 ml) which was washed with 320 ml of water and eluted with a linear gradient of ammonium carbonate from 0 to 0.3 M (6.4 liters). Fractions of 130 ml each were collected, and No. 20—No. 24 were evaporated to dryness (264 mg, 910 units/mg, 2.40×10^5 units).

c) The sample (264 mg) dissolved in 3 ml of water was applied to a QAE-Sephadex (OH) column (20 ml) which was washed with 80 ml of water and eluted with a linear gradient of ammonium carbonate from 0 to 0.57 M (1 liter). Fractions of 25 ml each were collected, and No. 13—No. 20 were evaporated to dryness (190 mg, 1100 units/mg, 2.09×10^5 units).

d) The sample (190 mg) dissolved in 10 ml of 0.1 M ammonium carbonate was applied to a QAE-Sephadex (CO_3) column (37 ml) equilibrated with 0.1 M ammonium carbonate and washed with 150 ml of the same solution. The factor was eluted with a 0.1 M to 0.4 M ammonium carbonate linear gradient starting with 750 ml of each solution. Fractions of 50 ml each were collcted, and No. 16—No. 19 were evaporated to dryness (Fig. 3b, 75 mg, 2500 units/mg, 1.88×10^5 units).

Step 9 (V). Preparative Paper Electrophoresis——The procedure taken here was almost the same as mentioned in Step 9 (IV) except voltage and time. The electrophoresis was carried out at 11.8 volts per cm for 3 hr. The first treatment resulted in 22 mg, 8300

units/mg, 1.83×10^5 units, and the second treatment, 17 mg, 10600 units/mg, 1.81×10^5 units.

Step 10 (V)——The sample (17 mg) dissolved in 4 ml of water was applied to a DEAE-Sephadex (HCO_3) column (7 ml) which was washed with 50 ml of water and eluted with a linear gradient of ammonium bicarbonate from 0 to 0.5 M (200 ml). Fractions of 6.25 ml each were collected, and No. 26—No. 29 were evaporated to dryness. The highly purified factor was changed to a calcium salt through a SE-Sephadex (Ca) column (0.4 mg, 310000 units/mg, 1.24×10^5 units). The electrophoretic character is shown in Table VI together with the factor from peak IV.

Discussion

A better recovery of activity was obtained in the present large scale procedure than the tentative purification system previously reported.[15] In step 2 a distinct difference was observed on the recovery of activity between free and acetate form of the weak basic resin as shown in Table II. This suggested an existence of some inhibitors in the free form resin-treated extract although a clear explanation for this has not been given. Amberlite IRA-68 in step 4 had the best usefulness for a rough separation of the acidic factors from a larger amount of impurities. It has the advantageous quality of adsorbing colored impurities which has been industrially applied to a refining process of sugar.[23] DEAE-Sephadex in step 7 was used for a fine separation of the acidic factors. This treatment first proved that there were at least four kinds of active acidic substances (peaks II to V) with the exception of peak I which probably contains a neutral factor. The 50% decreased recovery of total activity from step 6 to step 7 is still unexplained since the purification procedures in step 8 (IV) and (V) indicated satisfactory recoveries of activity under DEAE- or QAE-Sephadex column chromatography. But it is possible that the mixture of peak II, III, IV, and V may show a higher activity than each of them.

In all the steps described above, appreciable differences in specific activities were observed between the results of a laboratory scale procedure and those of a large-scale one. In general, the specific activities obtained from a large scale procedure were a half or one-third of those from a laboratory scale.

During the course of the purification procedure, a larger amount of L-malic acid as compared with trace amounts of other acids in the TCA-cycle and acidic amino acids was found in step 6 and step 4 respectively. In the acid hydrolyzed product of peak I in step 4 (Fig. 1a), glutamic acid occupied 27% of the original weight and aspartic acid 5%, and trace amounts of several other amino acids were found, while the sum of free glutamic acid and aspartic acid amounted to only 12% of the weight. This indicates that there are some acidic peptides mainly composed of glutamic acid in fractions of peak 1. Recently whole amino acid sequence of an acyl carrier protein, an acidic polypeptide, from *E. coli* was reported,[24] which contained much glutamic acid and aspartic acid, and had P-PaSH covalently linked to the hydroxyl group of the 36th serine residue from N-terminal. Since P-PaSH has a growth promoting activity for *B. bifidum* N4, the small activity of peak 1 might be due to something like acyl carrier protein from carrot.

In the case of DEAE-Sephadex chromatography (Fig. 2), P-PaSH appeared in close agreement with the fraction II. It is evident from the result that the main peak IV and the minor peak V does not coincide with P-PaSH or its disulfide. Moreover, both the paper

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24) T.C. Vanaman, S.J. Wakil, and R.L. Hill, *J. Biol. Chem.*, **243**, 6420 (1968); D.W. Majerus, A.W. Alberts, and P.R. Vagelos, *J. Biol. Chem.*, **240**, 4723 (1965); E.L. Pugh and S.J. Wakil, *J. Biol. Chem.*, **240**, 4727 (1965).

Table VII. *Rf* Values of Factors from IV and V, and P-PaSH on Thin-Layer Chromatograms[a)]

Solvent[b)]	I	II	III
Factor from IV (200000 units/mg)	0.22	0.65	0
Factor from V (310000 units/mg)	0.09	0.34	0.10
P-PaSH	0.28	0.48	0.16

a) adsorbent: crystalline cellulose (Avicel SF, Asahi Kasei, Co., Ltd).
b) solvent I, 1-butanol–AcOH–H_2O (5:2:3 v/v); II, 2-propanol–H_2O(3:1 v/v); III, 2-butyric aicd–0.5N NH_4OH (5:3 v/v)

electrophorograms (Table VI, Fig. 4) and the thin layer chromatograms (Table VII) clearly demonstrate the difference between P-PaSH and the factor of peak IV or V.

Moreover, CoA has a nearly equal mobility with P-PaSH in the paper electrophoresis (1 M formic acid). Other precursors of CoA and unsymmetrical disulfide of CoA and glutathione purified as a nucleotide peptide[25)] cannot explain such a large mobility of the factor from peak IV in the paper electrophoresis either. Therefore, it is apparent that the isolated factor from peak IV is a new compound. The following paper will describe a structural study of this factor.

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