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Bifidus Factors in Carrot. III.[1] The Structure of The Factor in Fraction V

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Molecular weight of the minor bifidus factor (III) in carrot was 700—800. III was converted to 3′-dephospho-coenzyme A (DP-CoA) with KCN or 2-mercaptoethanol. It suggested that III was 3′-dephospho-coenzyme A-S-sulfonic acid (DP-$CoASSO_3H$). This was confirmed by amino acid and phosphoric acid analysis and ultraviolet spectrum. Further synthesized DP-$CoASSO_3H$ coincided with III in behaviors of gel filtration, paper electrophoresis, thin-layer chromatography and ultraviolet spectrum.

Coenzyme A-S-sulfonic acid ($CoASSO_3H$) was also synthesized, and significances of these S-sulfonated compounds are discussed.

In the previous paper we identified the structure of the factor (I) in fraction IV with 4′-phospho-D-pantetheine-S-sulfonic acid (P-$PaSSO_3H$). This compound suggested wide distribution of S-sulfonate forms in nature and was also expected to propagate *Bifidobacterium bifidum* in intestines. In this paper we studied on the structure of the factor (III) in fraction V taking an analogous compound into consideration.

Result

In Gel Filtration, molecular weight of III was estimated as 700—800, since it was eluted with the void volume on Sephadex G-10, but eluted after coenzyme A (CoA) on Sephadex G-15 (Table I).

Table I. Gel Filtration on Sephadex G-15 in 0.1M $HCOONH_4$

Compound	M.W.[a]	Loaded dose	E.V.[b]	Detection
Blue dextran	2×10^6	0.3 mg	12 ml	absorbance at 650 mμ
CoA	768[c]	1 mg	13	absorbance at 259 mμ
DP-$CoASSO_3H$	768[c]	1 mg	14	bioassay and absorbance at 259 mμ
III		4000 units	14	bioassay
P-$PaSSO_3H$	469	1 mg	15	chemical reaction[3]
PaSS	555	10 mg	17	chemical reaction[3]
Glucose	180	10 mg	18	chemical reaction[4]

a) molecular weight *b*) elution volume *c*) free form

In paper electrophoresis III did not coincide with any known D-pantetheine (PaSH) derivatives. But when treated with KCN or 2-mercaptoethanol, III showed the same mobility as 3′-dephospho-coenzyme A (DP-CoA) (Fig. 1, 2).

The same behaviors were also observed in thin-layer chroamatography (Fig. 3, 4). These facts suggested that III was 3′-dephospho-coezyme A-S-sulfonic acid (DP-$CoASSO_3H$).

1) Part II: M. Yoshioka and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **19**, 178 (1971).
2) Location: *Hongo, Bunkyo-ku, Tokyo, 113, Japan.*
3) G. Toennis and J.J. Kolb, *Anal. Chem.*, 23, 823 (1951).
4) "Dyeing Reagents for Thin-Layer and Paper Chromatography," E. Merck,AG., Darmstadt, Germany, 1961.

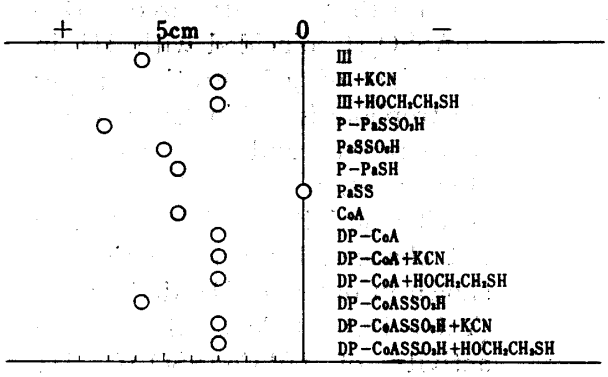

Fig. 1. Bioautogram of a Paper Electrophorogram on Toyo Roshi No. 51A Filter Paper in 1M HCOOH, pH 2 at 11.8 V/cm for 2 Hours

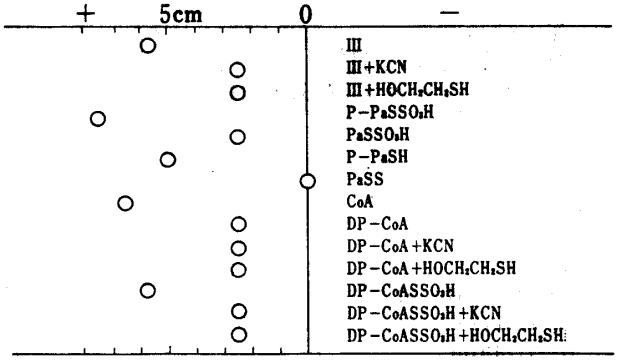

Fig. 2. Bioautogram of a Paper Electrophorogram on Toyo Roshi No. 51A Filter Paper in 0.1M NH_4HCO_3, pH 8 at 11.8 V/cm for 2 Hours

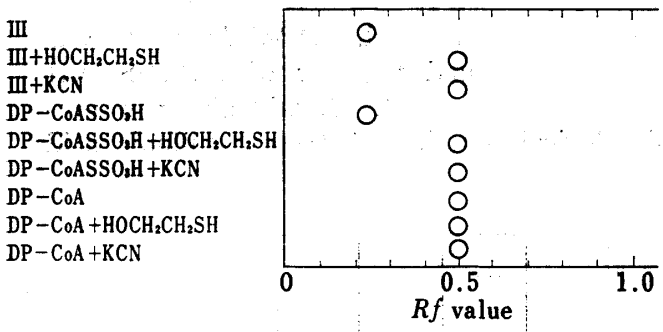

Fig. 3. Bioautogram of a Thin-Layer Chromatogram on Avicel SF developed with 1-Butanol–AcOH–H_2O (5:2:3 v/v)

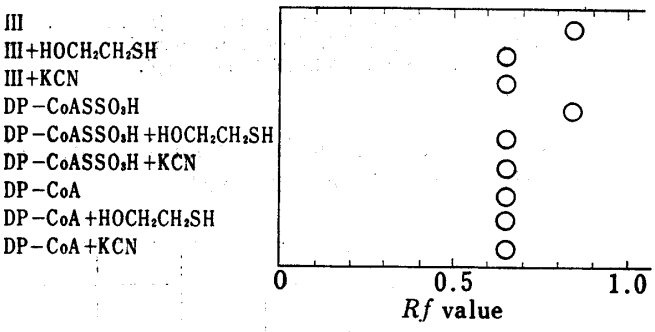

Fig. 4. Bioautogram of a Thin-Layer Chromatogram on Avicel SF developed with 5% Na_2HPO_4–2-Pentanol (1:1 v/v)

Table II. Determination of Amino Acid, Phosphoric Acid, Adenine of III and $CoASSO_3H$

Component\Sample	0.08 μmole of III[a]	0.06 μmole of $CoASSO_3H$
β-Alanine	0.08 μmole	0.062 μmole
Taurine	0.062	0.050
Glycine[b]	0.038	0.020
Phosphoric acid	0.162	0.180
Adenine[c]	0.083	0.060

a) Calculated from the microbiological activity based on the specific activity of 300 units/mμmole of DP-$CoASSO_3H$.
b) Probably due to degradation of adenine during oxidation and hydrolysis.
c) Calculated from UV absorption of III and $CoASSO_3H$ on the basis of molecular extinction coefficient of 1.5×10^4 in 0.01N HCl at 257 mμ.

In order to confirm this, we determined the components of III. As shown in Table II, one mole of β-alanine and taurine each and two moles of phosphoric acid were produced by performic acid oxidation, followed by hydrochloric acid hydrolysis of III.

These amino acids were also verified by dansylation (Fig. 5).

Ultraviolet (UV) maximum of III exibited the shift in hydrochloric acid characteristic of adenine (Fig. 6A).

Adenine in III was also identified by hydrochloric acid hydrolysis followed by thin-layer chromatography (Fig. 7).

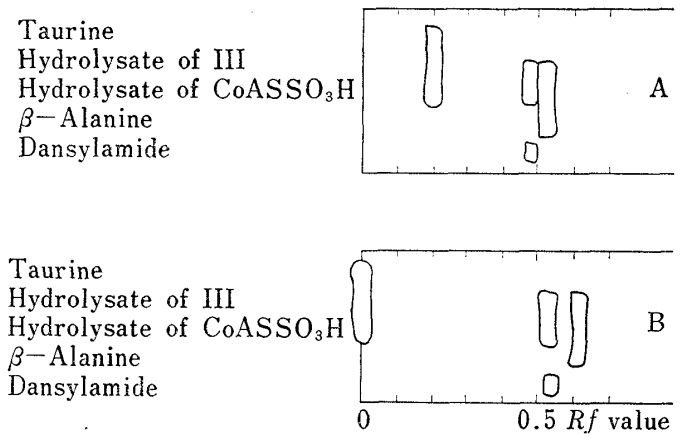

Fig. 5. Thin-Layer Chromatograms of Dansyl Derivatives of the Hydrolysates of III and $CoASSO_3H$ on Nylon Sheets

A was developed with H_2O–90% HCOOH (100:1.5 v/v). B with benzene–AcOH (9:1 v/v). The separated dansyl amino acids were visualized by ultraviolet irradiation (365 mμ).

Further, synthesized DP-$CoASSO_3H$ coincided with III in behaviors of gel filtration, paper electrophoresis, thin-layer chromatography and UV spectrum (Fig. 1—4, 6—7, Table I). The specific activity of DP-CoA was not changed by S-sulfonation as shown in other PaSH derivatives (Table III). Calculated from the activity of DP-$CoASSO_3H$, III was 85% pure.

Discussion

It is interesting that a new compound, DP-$CoASSO_3H$ was found together with P-$PaSSO_3H$ in carrot. It will give new problems about biosynthetic mechanism and phisyological function of these S-sulfonic acid derivatives of CoA precursors.[5]

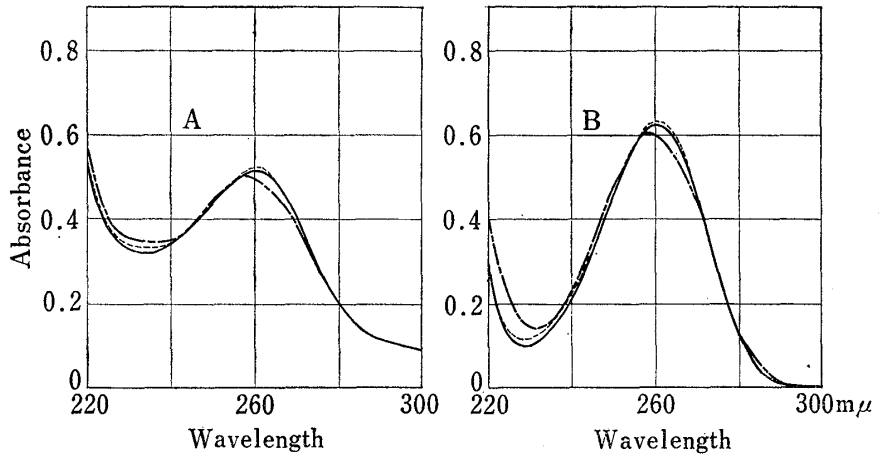

Fig. 6. UV Spectra of III and DP-$CoASSO_3H$

A: III (321 μg/10 ml). B: DP-$CoASSO_3H$ (39.5 μM). ——: in H_2O; ——: in 0.01N HCl, pH 2; ------: in 1N NH_4OH, pH 12.7

Experimental

The growth activity (unit) for *Bifidobacterium bifidum* N4 was determined by the method described in the previous paper. Paper electrophoresis, thin-layer chromatography, dansylation, treatments with KCN and 2-mercaptoethanol, and analysis of amino acid and phosphoric acid were also carried out in the same manner reported.[1] All the sample used for spotting corresponded to 40 units. For amino acid and phosphoric acid analysis 4×10^4 units of III and 126 μg (0.1 μmole) of $CoASSO_3H$ were used. Aqueous indophenol (1%) was used for detection of reducing compounds ($CoASSO_3H$). UV spectrum was measured with a Hitachi Recording Spectrophotometer Model EPS-2.

Gel Filtration on Sephadex——A column of Sephadex G-15 (1.5 × 20 cm) was bufferized with 0.1M HCOO-NH_4 (adjusted pH to 3 with HCOOH). The sample was dissolved in the same buffer, poured onto the column

5) M.B. Hoagland and G.D. Novelli, *J. Biol. Chem.*, **207**, 767 (1954); T. Suzuki, Y. Abiko, and M. Shimizu, *J. Biochem.*, **62**, 642 (1967).

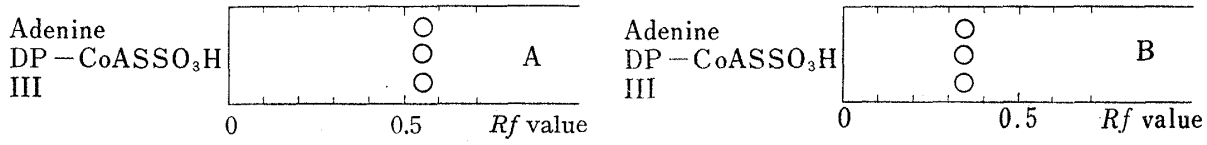

Fig. 7. Thin-Layer Chromatograms of the Hydrolysates of III, DP-CoASSO$_3$H and Adenine on Avicel SF

A: The upper layer of 1-butanol–pyridine–H_2O (6:3:2v/v)+pyridine (1v). B: The upper layer of 1-butanol–ethanol–H_2O (5:1:4 v/v). The separated adenine was visualized as a dark spot by ultraviolet irradiation (254 mμ).

Table III. Growth Activities of D-Pantetheine Derivatives

	Specific activity per 1 mμmole of PaSH		Specific activity per 1 mμmole of PaSH
PaSS	400 units	DP-CoA	300 units
PaSSO$_3$H	400	DP-CoASSO$_3$H	300
P-PaSH	400	CoA	200
P-PaSSO$_3$H	400	CoASSO$_3$H	200

and eluted with the buffer (Table I). Gel filtration of III on Sephadex G-10 was done in the same manner reported.[1]

Detection of Adenine——III (9000 units), DP-CoASSO$_3$H (1 mg) and adenine (1 mg) were dissolved in 0.2 ml of 1N HCl in test tubes, sealed *in vacuo* and heated at 85° for 2 hours. HCl was evaporated under a stream of N_2. The hydrolysates were dissolved in a small volume of water and spotted on thin-layers (Fig. 7).

Synthesis of Barium Salt of 3′-Dephospho-coenzyme A (DP-CoASSO$_3$H)——3′-Dephospho-coenzyme A·Li_2·5H_2O[8] of 79 mg (100 μmoles), Na_2SO_3·7H_2O of 126 mg (500 μmoles) and $CuSO_4$·5H_2O of 1.25 mg (5 μmoles) were dissolved in 20 ml of water. The solution was bubbled with air through water at room temperature overnight, and a poured onto a column of QAE-Sephadex (OH) (0.9×7 cm). The column was washed with water and eluted with a linear gradient of ammonium carbonate (0—0.5M) (total 200 ml). Fractions of 5.9 ml each were collected. The S-sulfonate was detected in No. 19—23 by UV absorption at 260 mμ and nitroprusside-KCN reaction.[3] These fractions were evaporated to dryness *in vacuo* below 40°. The same column chromatography was repeated once more. The S-sulfonate was converted to a barium salt through a column of SE-Sephadex (Ba) (10 ml). Yield, 65 mg (67%). *Anal.* Calcd. for $C_{21}H_{32}O_{16}N_7S_2Ba_{1.5}P_2$: C, 25.98; H, 3.33; N, 10.10; P, 6.38. Found: C, 25.90; H, 4.21; N, 10.34; P, 6.12. P: Adenine, 2.10:1. UV $\lambda_{max}^{H_2O}$ mμ (ε): 260.5 (15700). IR ν_{max}^{KBr} cm^{-1}: 3330, 2970, 2890, 1650, 1638, 1601, 1573, 1546, 1477, 1422, 1373, 1332, 1240, 1123, 1090—1020, 950.

Synthesis of Barium Salt of Coenzyme A-S-Sulfonic Acid (CoASSO$_3$H)——Coenzyme A·Li_3·3H_2O (83% purity, Sigma Chemical Co.) of 50 mg (50 μmoles), Na_2SO_3·7H_2O of 126 mg (500 μmoles) and $CuSO_4$·5H_2O of 1.25 mg (5 μmoles) were dissolved in 20 ml of water. The solution was bubbled with air through water at room temperature overnight, and poured onto a column of QAE-Sephadex (OH) (0.9×7 cm). The column was washed with water and eluted with a linear gradient of ammonium carbonate (0—1M) (total 200 ml). Fractions of 5.9 ml each were collected. The S-sulfonate was detected in No. 7—12 by UV absorption at 260 mμ and nitroprusside-KCN reaction.[3] These fractions were evaporated to dryness *in vacuo* below 40°. The same column chromatography was repeated once more. The S-sulfonate was converted to a barium salt through a column of SE-Sephadex (Ba) (10 ml). Yield, 59 mg (94%). *Anal.* Calcd. for $C_{21}H_{39}O_{23}N_7S_2Ba_{2.5}P_3$: C, 20.05; H, 3.13; N, 7.80; P, 7.39. Found: C, 20.23; H, 3.53; N, 7.71; P, 7.16. P: Adenine, 3.14:1. UV $\lambda_{max}^{H_2O}$ mμ (ε): 260.5 (15800). IR ν_{max}^{KBr} cm^{-1}: 3330, 2970, 2940, 2880, 1650, 1638, 1550, 1476, 1423, 1370, 1330, 1250—1175, 1112, 1090—1020, 935.

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