Studies on the Bacteria Isolated from Wine

Part V Isolation and Properties of the Growth Factor from Tomato Juice for a Bacterium Inducing Malo-lactic Fermentation

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The growth factor for malo-lactic fermentation bacteria was partially purified from tomato juice, and some properties of the factor were examined. The factor was comparatively a low molecule, thermo-stable and insoluble in nonpolar organic solvents. The purified factor fraction showed positive reaction with Molisch-, tetrazolium- and Nessler-reagents, suggesting the existence of sugar and nitrogen as the chemical components. The treatment with cellobiase or cellulase-II resulted in the inactivation of the factor and simultaneously released the sugar constituent composed of glucose from the factor. These results indicated that the glucose residue might exist as the β -1,4-glucosidic linkage in the growth factor.

In the previous paper¹⁾ the distribution of the factor, which was necessary for the growth of malo-lactic fermentation (MLF) bacteria strain WNB-75, has been examined over varieties of plants and animal tissues. It was found that this growth factor distributed widely in various plants, particularly those belonging to the fruits and vegetables such as grape, citrus, tomato, *ect.* The content of the factor in grape was varied depending upon varieties, degree of ripeness and harvest time. Thus, it seems likely that the content of the factor in grape used as raw materials for wine making correlates the occurrence of MLF in wine.

In order to elucidate the physiological significance of the factor in the MLF bacteria, it is important to isolate the factor and make clear its chemical character. The present paper deals with the procedures of partial purification from tomato juice (TJ) and some properties of the factor.

EXPERIMENTAL AND RESULTS

I. Assay method of the activity of the growth factor

The activity of the growth factor was measured by photometrical growth response of strain WNB-75 under the culture conditions as previously described.¹⁾

II. Isolation of growth factor from TJ

a) Some properties of the factor in crude concentrated extract

Preceding to the separation of the factor, some chemical and physical properties were investigated as follows.

1. *Dialytic property.* The factor could easily permeate through a cellophane membrane.

2. *Stability for light.* The factor was not inactivated after 60 hr under the sunlight at pH 2.0~13.0.

3. *Stability for heat.* The factor was not completely decomposed in 1N hydrochloric

1) H. Yoshikumi and T. Amachi, *Agr. Biol. Chem.*, **33**, 18 (1969).

acid- or 2N sodium hydroxide-solution at 100°C for 1 hr and it could also be autoclaved with basal medium without any loss in activity (1 kg/cm², 5 min).¹¹ After the preparation of the factor was reduced to ash, the activity was lost completely, suggesting that the factor is organic or volatile compound.

4. *Stability for oxidants.* The factor was inactivated after incubation at 25°C for 24 hr with 5% of hydrogen peroxide or 1N potassium permanganate.

5. *Solubility in organic solvents.* The factor was freely soluble in water, passably soluble

TABLE I. THE ISOLATION PROCEDURES OF THE GROWTH FACTOR FROM TOMATO JUICE

Procedure	Dry weight (g)	Specific activity of factor (u/g)	Total activity
Tomato juice			
Centrifuged			
Residue			
Tomato juice serum	51,040	78	4.0×10^6
Absorbed on active charcoal			
Eluted by acetone			
Concentrated <i>in vacuo</i>			
Concentrated eluate	20,580	258	5.3×10^6
Added EtOH (final conc. 70%)			
Stood over night at 0°C			
Precipitate			
Removed EtOH <i>in vacuo</i>			
Concentrated filtrate	6320	665	4.2×10^6
Passed through Amberlite			
CG-120 column			
Eluate	5950	588	3.5×10^6
Chromatographed on active charcoal			
Eluate (eluted by 20% EtOH)	613.2	57,100	3.5×10^7 *
Chromatographed on Dowex 1, $\times 8$			
Eluate (eluted by 0.05N HCOOH)	36.4	7.0×10^5	2.5×10^7
Chromatographed on DEAE-Sephadex, A-25			
Eluate (eluted by 0.25% (NH ₄) ₂ CO ₃)	3.7	3.0×10^5	1.1×10^7
Desalted by Amberlite CG-120			
Eluate	0.55	2.0×10^7	1.1×10^7

* In this step the phenomenon of the increase in total activity seems to be due to the removal of inhibitory components against strain WNB-75.

in ethanol or methanol, and insoluble in ether, chloroform or ethyl acetate.

b) Procedures of isolation

1000 liters of TJ was prepared for the isolation of the growth factor. The procedures of isolation are summarized in Table I. TJ serum (800 liters) was obtained from the raw juice by centrifuge. Active charcoal (24 kg) was added into the TJ serum at pH 4.2 at 25°C and the serum was stirred for 1 hr followed by separation of charcoal. The serum filtrate was treated repeatedly with 3% (w/v) of active charcoal and after three times treatments, no growth factor activity was found in the filtrate. Charcoal was combined and extracted with five volumes of acetone. Acetone extracts were evaporated to dryness *in vacuo* and dissolved in 70% solution of ethanol and then allowed to stand over night at 0°C. After removing the resulting precipitate (not active) by filtration, the filtrate was concentrated *in vacuo* and passed through the column of Amberlite CG-120 resin (H^+ form). The factor was not absorbed on the resin under the conditions used. The effluent was subjected to chromatography of a charcoal column.

FIG. 1. Charcoal Column Chromatography of the Growth Factor.

Column: Active charcoal (for chromatography, Wako) 55 mm $\times$ 450 mm
One fraction: 1000 ml
Eluant: 0~30% EtOH (stepwise)
Elution rate: 20 ml/min

FIG. 2. Ion Exchange Chromatography of the Growth Factor.

Column: Dowex-1, $\times 8$, formate form, 10 mm $\times$ 200 mm
One fraction: 5 ml
Eluant: 0.01~0.1N formic acid (gradient)
Elution rate: 1 ml/min

FIG. 3. Column Chromatography of the Growth Factor on DEAE Sephadex.

Column: DEAE Sephadex, A-25, 0.05% $(NH_4)_2CO_3$ bufferized, 26.5 mm $\times$ 400 mm
One fraction: 10 ml
Eluant: 0.05~0.25% $(NH_4)_2CO_3$
Elution rate: 2 ml/min

The elution pattern of the factor is illustrated in Fig. 1. The active fraction was subsequently chromatographed on a Dowex-1, $\times 8$ column (formate form). When the factor solution was adjusted at pH 10.0 by sodium hydroxide, the factor was completely absorbed on this resin. The elution was carried out with a linear gradient increase in the concentration of formic acid from 0.01 to 0.1N. The factor was eluted in the vicinity of 0.05N formic acid as shown in Fig. 2. The effluent containing the factor

was chromatographed on DEAE Sephadex (A-25, bufferized with 0.05% ammonium carbonate). The factor was eluted by 0.25% ammonium carbonate as shown in Fig. 3. The active fractions were collected and desalted by Amberlite CG-120 (H^+ form).

In the final step the activity of the factor was attained to 2.5×10^5 fold-increase per dry weight, and 0.05 μg of the purified substance per ml of culture medium was active as the growth factor for strain WNB-75.

c) Thin layer chromatography of the factor

Thin layer chromatography (TLC) was attempted with the active fraction of the final step of the isolation procedure. The method and results of the chromatography were sum-

marized in Table II. The detection of the factor on the chromatogram was carried out by the bioassay method. Chromatographic analyses suggested the factor to be one component, because the factor fraction on these chromatograms gave always a single spot in different solvent systems. The factor gave a spot on the chromatogram by spraying iodine vapor, anisaldehyde-sulfuric acid and naphthoresorcinol-phosphoric acid. The further purification of the factor was carried out by TLC with solvent systems C, H, J and L (Table II), and this preparation of the factor was used in the following experiments. The purified preparation of the growth factor showed no absorption peak between 220 and 320 $m\mu$.

TABLE II. THIN LAYER CHROMATOGRAPHY OF THE GROWTH FACTOR ON KIESELGEL G PLATES

Solvent system	R _f value
a) Acetic acid, Pyridine, Water (5 : 1 : 4)	0.93
b) Methylene chloride, MeOH, Formamide (80 : 19 : 1)	Not obvious
c) Methyl acetate, iso-PrOH, 25% NH ₄ OH (45 : 35 : 20)	0.12
d) Chloroform, 96% Acetic acid (95 : 5)	0.02
e) Chloroform, MeOH, 96% Acetic acid (75 : 20 : 5)	0.20
f) <i>n</i> -BuOH, Acetic acid, Water (60 : 20 : 20)	0.56 (tailing)
g) <i>n</i> -PrOH, 28% NH ₄ OH (67 : 33)	Not obvious
h) <i>n</i> -BuOH, Acetic acid, 5% NH ₄ OH (55 : 30 : 15)	0.54
i) Phenol, Water (75 : 25)	Not obvious
j) Benzene, MeOH, Acetone, Acetic acid (70 : 20 : 5 : 5)	0.21
k) Acetone, Dioxane, 25% NH ₄ OH (45 : 45 : 10)	0.02
l) <i>n</i> -BuOH, iso-PrOH, Water (50 : 25 : 20)	0.18

Reagents for detection of spot

1) I_2 in chloroform

2) Anisaldehyde- H_2SO_4 in EtOH

3) Naphthoresorcinol- H_3PO_4

Kieselgel G was activated by heating 110°C for 50 min.

III. Some properties of the purified preparation of the factor

a) Qualitative reactions

Some spot tests²⁾ of elements and tests for functional groups³⁻⁸⁾ were carried out with the purified preparation of the factor (Tables III, IV). The preparation gave positive reaction of nitrogen, but not of sulfur or phos-

TABLE III. SPOT TESTS OF THE FACTOR

Tested element	Fundamental method ²⁾	Result
N	Ignited with CaO and MnO ₂ , and detected by Ag-Mn and benzidine	+++
S	Heated with HCOONa, and detected by lead acetate	—
P	Ignited with CaO, and detected by ammonium molybdate and benzine	—

2) F. Feigl, "Spot Tests in Organic Analysis," Elsevier Publishing Co., 1960, p. 95.

3) A. Weissberger, "Technique of Organic Chemistry," Vol. 6, Interscience Publishers, 1954, p. 447.

4) F. Feigl, "Spot Tests in Organic Analysis," Elsevier Publishing Co., 1960, p. 119.

5) F. Feigl, *ibid.*, p. 236.

6) L. A. Elson and W. T. J. Morgan, *Biochem. J.*, **28**, 988 (1934).

7) C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).

8) I. Mori and S. Kobayashi, "Practical Method in Paper-electrophoresis" (Japan), Nankodo Press, 1955, p. 40.

TABLE IV. QUALITATIVE COLOR REACTIONS OF THE FACTOR

Tested reaction	Result	Tested reaction	Result
Molisch ³⁾	±	Nitroprusside ⁵⁾	—
Fehling ³⁾	—	Elson-Morgan ⁶⁾	±
Benedict ³⁾	—	Fiske-Subbarow ⁷⁾	—
Tetrazolium ³⁾	±	FeCl ₃ ⁸⁾	—
Ninhydrin ³⁾	—	LiAlH ₄ ⁸⁾	—
Nessler ⁴⁾	+		

phrous element. It gave positive reaction with Nessler-reagent⁴⁾ and was slightly positive in reducing reactions by Molisch³⁾ and tetrazolium reagents,³⁾ or by amino sugar reagent of Elson-Morgan.⁶⁾

b) *The changes in biological activity by chemical and enzymatic treatments*

The purified preparation, 0.2 ml (50 µg/ml), was hydrolysed with acid or alkali at various concentration (final concentration 1N to 6N). After hydrolysis at 100°C for 8 hr in a water bath, the solutions were neutralized and desalted with Amberlite CG-120 (H⁺ form) and CG-400 (Cl⁻ form). The hydrolyzed product(s) was assayed for the activity of the factor and simultaneously tested with some reagents of qualitative reaction. The results were shown in Table V. The factor became inactive by hydrolysis with 2N hydrochloric acid at 100°C for 8 hr, but by 2N sodium hydroxide slight activity remained. In 6N sodium hydroxide solution the factor became completely inactive, followed by a remarkable increase in positive

reaction with Molisch, tetrazolium, or Nessler reagent. These results suggest that the factor can be degraded by acid or base and contains saccharide and nitrogen component in its structure. Saccharide content was measured as glucose with anthrone method.⁹⁾ Nitrogen component in the factor was decomposed with conc. sulfuric acid and determined as ammonium by Nessler reaction.¹⁰⁾ The factor was found to contain 9.5% of sugar and 3.71% of nitrogen on the dry weight basis.

Further proof of the sugar residue in the factor was obtained by the use of a few glucosidases. 50 µg of the crude naringinase,¹¹⁾ which splits naringin glucosidic linkage, was added to 5 ml (500 µg of dry weight, pH 5.0) of the factor solution. The reaction was carried out at 40°C for 2 hr. After the enzymatic reaction, increased reducing sugar was determined by copper reagent.¹²⁾ As shown in Table VI, treatment of the factor with the naringinase resulted in complete loss in the activity of the factor, accompanying by the increase of the reducing sugar that was identified to be glucose by TLC. It was concluded that the glucosidic linkage present in the structure of the factor was split by the enzyme to cause the loss of the activity of the factor.

To determine the type of the glucosidic linkage the purified preparation of the factor was treated with several glycosidases, and the changes in the activity were examined. The enzymes employed in this experiment were

TABLE V. THE EFFECT OF ACID OR ALKALI TREATMENT ON THE FACTOR

Treatment	Activity of factor	Molisch reaction	Tetrazolium reaction	Nessler reaction
No treatment	##	±	+	+
In 1N HCl, 100°C, 8 hr	##	±	+	+
In 2N HCl, 100°C, 8 hr	—	±	+	##
In 6N HCl, 100°C, 8 hr	—	±	+	+
In 1N NaOH, 100°C, 8 hr	##	±	+	+
In 2N NaOH, 100°C, 8 hr	+	±	+	+
In 6N NaOH, 100°C, 8 hr	—	##	##	##

9) F. A. Loewus, *Anal. Chem.*, **24**, 219 (1952).

10) G. L. Miller and E. E. Miller, *Anal. Chem.*, **20**, 481 (1948).

11) S. Okada, K. Kishi, M. Higashihara and J. Fukumoto, *Nippon Nogeikagaku Kaishi*, **37**, 84 (1963).

12) M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).

TABLE VI. THE EFFECT OF NARINGINASE
TREATMENT ON THE FACTOR

Treatment	Activity of factor	Reducing sugar ($\mu\text{g}/\text{ml}$, as glucose)
Factor (no treat)	≡	35
Factor + naringinase (40°C, for 2 hr)	—	85
Factor + inactivated naringinase* (40°C, for 2 hr)	≡	35
Controls		
{ Naringinase	—	0
{ Inactivated naringinase*	—	0

* Inactivated by heat-treatment (100°C, for 20 min)

the gluco-amylase,¹³⁾ cellobiase,¹⁴⁾ cellulase-I,¹⁴⁾ cellulase-II¹⁴⁾ and hemicellulase,¹⁵⁾ each of which was proved to be electrophoretically homogeneous. An appropriate amount of each enzyme (1 ml) was added to 1 ml of the factor solution (500 $\mu\text{g}/\text{ml}$) and incubated at pH 4.5 and 40°C. After incubation for 10 hr, the remaining activity of the factor and the amount of reducing sugar were determined. The activity of the factor was completely lost by the action of cellobiase or cellulase-II as shown in Table VII. In the case of cellobiase or cellulase-II treatment, the reducing sugar of the factor was increased from 35 μg to 85 μg or 90 μg , as glucose per 1 mg of the factor in dry weight

FIG. 4. Thin Layer Chromatogram of the Hydrolysate of the Growth Factor by Cellobiase.

- 1) Authentic glucose
 - 2) Authentic cellobiose
 - 3) Growth factor purified with TLC
 - 4) Cellobiase
 - 5) Cellobiose + cellobiase* (not reacted)
 - 6) Cellobiose + cellobiase (reacted for 10 hr)
 - 7) Growth factor + cellobiase* (not reacted)
 - 8) Growth factor + cellobiase (reacted for 10 hr)
- * Inactivated by heat-treatment (100°C, for 20 min)

Thin layer plate: Kieselgel G, activated for 50 min at 110°C

Solvent system: *n*-BuOH, iso-PrOH, Water (50 : 25 : 20)Reagent: Anisaldehyde-H₂SO₄

respectively. The reducing sugar liberated from the factor was identified as glucose comparing its *R_f* values on TLC of various solvent systems with that of authentic sample. Figure 4 showed the thin layer chromatogram of the hydrolysate of the factor by cellobiase.

TABLE VII. THE EFFECT OF GLUCOSIDASE ON THE FACTOR

Glucosidase	Enzyme activity* (u/ml)	Remained activity of factor	Decomposing linkage-type	Reducing sugar ($\mu\text{g}/\text{mg}$, as glucose)
None	—	≡	—	35
Gluco-amylase	800	≡	α -1,4, α -1,6	35
Cellobiase	194	—	β -1,4	85
Cellulase-I	85	≡	β -1,4 (high molecular)	35
Cellulase-II	133	—	β -1,4	90
Hemicellulase	330	≡	Pentose polymer	35

* One unit of enzyme activity was defined as the enzyme quantity which liberated 1 mg of glucose per minute.

One ml of each enzyme solution was added to 1 ml of the factor solution and incubated for 15 hr at pH 4.5 and 40°C.

13) Y. Tsujisaka and J. Fukumoto, *Science and Industry*, **28**, 2 (1954).14) Y. Tsujisaka and J. Fukumoto, *ibid.*, **37**, 494 (1963).15) J. Fukumoto, Y. Tsujisaka, S. Okada, M. Higashihara, S. Takenishi and S. Kato, *The Ann. Meet. Agr. Chem. Soc. Japan* (1966).

c) *Gas-liquid chromatography of the hydrolysate of the factor*

The liberated sugar from the factor by cellobiase hydrolysis was also identified with gas-liquid chromatography (GLC). The samples were converted to trimethylsilyl derivatives according to the method of Sweeley *et al.*¹⁶⁾ and detected by the use of Hitachi gas chromatograph K-53 equipped with a hydrogen flame ionization detector. GLC operations were all the same as Brobst and Lott's method.¹⁷⁾ The chromatogram obviously showed that glucose was liberated from the factor by the action of cellobiase (Fig. 5).

FIG. 5. Gas Liquid Chromatogram of the Factor Hydrolysate by Cellobiase.

Sample: (A) Factor preparation (trimethylsilyl-derivatized). (B) Factor hydrolysate by cellobiase (trimethylsilyl-derivatized)

Column: 3% silicone (SE-52) on Chromosorb W, 2 m (stainless steel)

Column temperature: Linear temperature programming from 140°C to 300°C (10°C/min)

16) C. C. Sweeley, R. Bentley, M. Makita and W. W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).

17) K. M. Brobst and C. E. Lott, Jr., *Cereal Chem.*, **43**, 35 (1966).

DISCUSSION

The isolation procedure of the growth factor for strain WNB-75 and some chemical properties of the purified growth factor are shown in the present paper. Many bacterial growth factors has been studied, but the TJ growth factor for strain WNB-75 differs from others in some chemical properties. In fact, known vitamins but calcium pantothenate, amino acids or nucleic acids in 1~1000 $\mu\text{g/ml}$ of medium were not effective for the growth of strain WNB-75. Only pantothenate was found to stimulate the bacterial growth, but chemical properties of pantothenate distinctly differ from those of the factor. The effect of pantothenate and its related compounds on the growth of strain WNB-75 will be reported in the next paper of this series.

Pollack and Lindner¹⁸⁾ (1943) noticed that the growth factor for *L. casei* was present in extracts of animals or plant tissues, and that it was soluble in water, but not in some organic solvents, and had heat stability at the pH values between 2 and 11. Wooley¹⁹⁾ (1946) studied the growth factor for *L. casei* in tryptic hydrolysate of casein, and found it to be the peptide named streptogenin. But casein tryptic hydrolysate was not active for the growth of strain WNB-75. Metcalf *et al.*²⁰⁾ (1946) investigated a growth factor for some *Lactobacilli* in vegetable juices, and showed that thiamine could be substituted for the growth factor, which also differs from the WNB-75 factor in heat stability and other properties. Shorb²¹⁾ (1947), Sauberlich and Baumann²²⁾ (1948), Williams *et al.*²³⁾ (1949), Smith²⁴⁾ (1949), and

18) M. A. Pollack and M. Lindner, *J. Biol. Chem.*, **147**, 183 (1943).

19) D. W. Wooley, *ibid.*, **162**, 383 (1946).

20) D. Metcalf, G. J. Hucker and D. C. Carpenter, *J. Bacteriol.*, **51**, 381 (1946).

21) M. S. Shorb, *J. Biol. Chem.*, **169**, 455 (1947).

22) H. E. Sauberlich and C. A. Baumann, *ibid.*, **176**, 165 (1948).

23) W. L. Williams, E. Hoff-Jorgensen, and E. E. Snell, *ibid.*, **177**, 933 (1949).

24) F. R. Smith, *J. Bacteriol.*, **45**, 369 (1949).

Snell *et al.*²⁵⁾ (1950) found an unknown bacterial growth factor in liver extracts, casein hydrolysates, yeast extracts, tomato juice or others, but none of them elucidated its properties. Hendlin *et al.*²⁶⁾ (1950) made it clear that the growth accelerating factors for *L. lactis* in tomato juice were pyridoxal phosphate and pyridoxamine phosphate, which were ineffective on the growth of strain WNB-75. Kodama^{27,28)} (1956) studied the growth factor for *L. fructosus* in tomato juice and found that fructose and nicotinamide were required for its growth. But nicotinamide was ineffective on the growth of strain WNB-75. Tomarelli *et al.*²⁹⁾ (1954) isolated the growth factor for *L. bifidus* from gastric juice of pig, and presumed it as galactose-acetylglucosaminide. Lambert and Zilliken³⁰⁾ (1965) noticed that D-glucosamine derivatives besides N-acetylglucosamine had activity for the growth of *L. bifidus*, and they proved that these compounds were incorporated to the bacterial cell wall. However, no activity as a growth factor for strain WNB-75 was found with 1 μ g to 1000 μ g

of D-glucosamine or N-acetyl-D-glucosamine per ml of the medium.

Hydrolysis by unpurified naringinase preparation showed that the factor was a suitable substrate and inactivated by the enzyme. Naringin is a flavanone glucoside containing glucose and rhamnose. The ultraviolet absorption spectrum of the purified factor fraction eliminated the existence of flavone ring. Moreover, the factor was negative in ferric chloride and lithium aluminium hydride reactions. The factor, thus, seems not to be flavanone glucosidic compound. Cellobiase and cellulase-II treatment also made the factor inactive. Cellobiase can attack oligosaccharides containing β -1,4-glucosidic linkage such as cellobiose. On the other hand, cellulase-II hydrolyzes β -1,4-oligosaccharides of comparatively low molecular weight in cellulose hydrolysates. These results definitely indicate that the growth factor for strain WNB-75 has glucose residue containing β -1,4-glucosidic linkage, and that the activity of the factor was lost with the cleavage of the linkage by the action of β -glucosidases.

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26) D. Hendlin, M. C. Caswell, V. J. Perters and T. R. Wood, *J. Biol. Chem.*, **186**, 647 (1950).

27) R. Kodama, *Nippon Nogeikagaku Kaishi*, **30**, 219 (1956).

28) R. Kodama, *ibid.*, **30**, 224 (1956).

29) R. M. Tomarelli, J. B. Hassinen, E. R. Eckhardt, R. H. Clark and F. W. Bernhart, *Arch. Biochem. Biophys.*, **48**, 225 (1954).

30) R. Lambert and F. Zilliken, *ibid.*, **110**, 544 (1965).

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