Biosci. Biotech. Biochem., **61** (10), 1705-1709, 1997

Purification of Levan Fructotransferase from *Arthrobacter nicotinovorans* GS-9 and Production of DFA IV from Levan by the Enzyme

Katsuichi SAITO, Hiroko GOTO, Atsushi YOKOTA, and Fusao TOMITA†

Laboratory of Applied Microbiology, Department of Bioscience and Chemistry, Faculty of Agriculture, Hokkaido University, Sapporo 060, Japan

Received April 21, 1997

A bacterial strain, GS-9, isolated from soil as a levan-degrading microorganism produced an extracellular enzyme that converted levan into DFA IV. This strain was identified as *Arthrobacter nicotinovorans*. The DFA IV-producing enzyme was specifically induced by levan. The enzyme was purified 60-fold from culture supernatant to give a single band on SDS–PAGE. The molecular weight of this enzyme was 52,000 by SDS–PAGE and a monomer by gel filtration. The enzyme gave DFA IV as a main product (>75%), and fructose, levanbiose, and two unidentified oligosaccharides as minor products, and was identified as a novel levan fructotransferase.

Key words: levan; levan fructotransferase; DFA IV; *Arthrobacter nicotinovorans* GS-9

Levan is a β-2,6-linked fructan that is found as a reserve carbohydrate of such monocotyledons as ryegrass and cocksfoot. Levan is also easily produced by several kinds of bacteria[1] during their assimilation of sucrose through the action of levansucrase (EC 2.4.1.10). For effective use of unused natural polysaccharides such as levan, we have been studying the effective production of useful oligosaccharides from these polysaccharides by microbial enzymes. We have efficiently produced di-D-fructose-1,2′:2,3′-dianhydride (DFA III) from inulin, a β-2,1-linked fructan, by inulase II (EC 2.4.1.93) of *Arthrobacter* sp. H65-7,[2,3] inulotriose from inulin by an inulin degrading enzyme of *Streptomyces rochei* E87,[4] and levanbiose from levan by a levan-degrading enzyme (LDE) of *Streptomyces exfoliatus* F3-2.[5] It has been reported that there are three types of microbial LDEs, *i.e.*, levanase (EC 3.2.1.65),[6] 2,6-β-D-fructan 6-levanbiohydrolase (EC 3.2.1.64), which splits levan into levanbiose,[5,7] and levan fructotransferase of *Arthrobacter ureafaciens* which produces di-D-fructose-2,6′:6,2′-dianhydride (DFA IV) from levan through intramolecular transfructosylation.[8,9] Another enzyme of *Arthrobacter* sp. No. 11-E involved in the formation of DFA IV from levan[10] has also been reported.

It has been shown that oligosaccharides have various physiological functions,[11] and DFA IV would be expected to have such functions. However, studies on physiological functions of oligosaccharides, including DFA IV, have not been extended due to limitations of their availability. Thus, we have started the screening of microorganisms for the effective production of DFA IV, and we have isolated a bacterium, that effectively produced an extracellular DFA IV-producing LDE, *i.e.*, levan fructotransferase.

In this paper, we describe identification of the microorganism and culture conditions for the levan fructotransferase production, and also purification and properties of the levan fructotransferase for the effective production of DFA IV.

Materials and Methods

Media. LA medium consisted of 5 g of yeast extract, 10 g of polypeptone, 10 g of NaCl, 20 g of agar, and NaOH to adjust the pH to 7.0, per liter. For the production of the DFA IV-producing LDE, LDE production medium[5] was used. LDE production medium consisted of 10 g of levan, 2 g of NH_4NO_3, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 0.5 g of KH_2PO_4, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.2 g of yeast extract, 20 g of $CaCO_3$, and NaOH to adjust the pH to 7.0, per liter. These media were sterilized at 121°C for 10 min.

Chemicals. Levan was prepared from *Serratia levanicum*[12] by the method of Yokota *et al.*[5] Inulin (from chicory root) was purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. Yeast extract was a product of Oriental Yeast Co., Ltd., Tokyo. Polypeptone was a product of Nihon Pharmaceutical Co., Ltd., Tokyo. Levanbiose was prepared by *Streptomyces exfoliatus* F3-2[5] and other levanoligosaccharides were purified from partial-HCl hydrolysates of levan by activated charcoal column chromatography. Inulobiose and DFA III were prepared by *Arthrobacter* sp. H65-7.[2,13] Molecular weight markers of protein were purchased from Boehringer Mannheim GmbH, Mannheim, Germany.

Analytical methods. TLC was done by method B as described previously.[3] The reducing power of the compound was checked by the Somogyi–Nelson method.[14] Sugars in the culture broth were measured by phenol–sulfuric acid method[15] using levan as a standard. DFA IV produced in the culture broth or reaction mixture was measured by HPLC with a refractive index detector (column, YMC-Pack ODS-AQ 6 × 250 mm; mobile phase, water). Growth was measured by the optical density of culture broth at 600 nm. Protein was measured by the method of Lowry *et al.*[16] SDS–PAGE was done on 10% gel by the method of Laemmli.[17]

Screening and identification of DFA IV-producing microorganisms. Screening of LDE-producing microorganisms was by the method of Yokota *et al.*[5] Among about 50 strains which had the DFA IV-producing LDE, strain GS-9 was chosen as the best producer of DFA IV. The strain was identified by the methods of Kodama *et al.*[18] and of Ochiai and Kawamoto.[19]

Preparation of crude LDE. Crude LDE was prepared as follows. One loopful of the bacterium cultivated on LA slant at 27°C for 2 d was inoculated into 100 ml of LDE production medium in a 500-ml flask, and then cultivated with shaking (120 rpm) at the same temperature for 24 h. The culture broth was centrifuged at 2000 × *g* at 4°C for 20 min, and the supernatant was used as the crude LDE.

Isolation and identification of DFA IV. The reaction mixture, which

† Corresponding author.

Abbreviations: DFA IV, di-D-fructose-2,6′:6,2′-dianhydride; LDE, levan-degrading enzyme; DFA III, di-D-fructose-1,2′:2,3′-dianhydride.

contained 100 ml of the crude LDE and the same volume of levan (20 mg/ml) in 0.1 M sodium phosphate buffer (pH 6.0), was incubated at 37°C for 48 h. The mixture was heated for 5 min in boiling water to stop the reaction, and was concentrated under reduced pressure. The resultant concentrate was put on an activated charcoal column (2.6 × 100 cm) washed with water. Sugars in the column were eluted with water. The fractions (20 ml each) were analyzed for the sugar by TLC, and sugar-containing fractions were combined, concentrated under reduced pressure, and lyophilized. The molecular weight was taken from the molecular-ion peak of the FD-MS measured by a JEOL JMS-SX 102A. The ^{13}C-NMR chemical shifts were recorded with a Bruker AM-500 with complete proton decoupling.

Assay of DFA IV-producing activity. The DFA IV-producing activity was assayed using a reaction mixture containing 50 mM sodium phosphate buffer (pH 6.0), levan (10 mg/ml), and the enzyme solution in a total volume of 0.5 ml. The mixture was incubated at 37°C for 10 min, and then heated in boiling water for 5 min to stop the enzyme reaction. DFA IV was measured by HPLC. One unit of enzyme activity was defined as the amount of the enzyme that produced 1 μmol of DFA IV per min under these assay conditions.

Purification of the DFA IV-producing LDE. The culture supernatant (1400 ml) was concentrated in cellulose tubes with polyethylene glycol 20,000 at 4°C for several hours. To the concentrated solution (200 ml), solid ammonium sulfate was added to give 60% saturation. The precipitate formed was collected, dissolved in 10 ml of 10 mM sodium phosphate buffer (pH 7.0), and dialyzed against equilibration buffer for column chromatography as follows. Anion-exchange column chromatography was done on a DEAE-Toyopearl 650M column (1.9 × 14 cm) (Tosoh Co., Ltd., Tokyo) equilibrated with 10 mM sodium phosphate buffer (pH 7.0). The enzyme was eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer. The active fractions were combined and concentrated by ultrafiltration (Centriplus 10, Amicon, Inc.). Gel chromatography was done on a Bio-Gel P-100 column (2.2 × 78 cm) (Bio-Rad Laboratories). The buffer for equilibration and elution was 10 mM sodium phosphate buffer (pH 7.0) containing 0.2 M NaCl. For estimation of molecular weight, a Bio-Gel P-150 column (2.6 × 73 cm) was used instead of a Bio-Gel P-100 column.

Results and Discussion

Identification of the strain GS-9

Strain GS-9 was found to be a Gram-positive, non-spore forming bacterium, which had a rod-coccus growth cycle, and had no *meso*-diaminopimelic acid in its cell wall hydrolysate. It had the peptidoglycan type of Lys-Ala-Thr-Ala. Morphological characteristics and partial 16S rDNA of this strain showed high similarity with *Arthrobacter nicotinovorans*. From two-dimensional electrophoresis of ribosomal proteins of strain GS-9 and *A. nicotinovorans* KY3215, SAB values between the two strains were over 0.90 in each region. Therefore, the strain GS-9 was identified as *Arthrobacter nicotinovorans* GS-9. The details of these taxonomic study will be published elsewhere.

Table I. ^{13}C-NMR Chemical Shifts of the Purified Product

Assignment (Carbon atom number)	Chemical shifts[a] of ^{13}C-NMR of	
	Product	DFA IV[b]
1	60.7	60.7
2	103.5	103.5
3	81.2	81.2
4	77.2	77.4
5	72.1	72.3
6	59.6	59.8

[a] δ in ppm at 125 MHz with TMS as an external standard in D_2O.
[b] Data are from ref. 20.

Identification of the reaction products

The reaction products after boiling for 5 min were analyzed by TLC. A main compound was found between fructose and levanbiose in the mobility on TLC, and fructose was found as a minor product. This main product was purified and analyzed as described in Materials and Methods. It had no reducing power and its molecular weight was 324. Table I shows the ^{13}C-NMR chemical shifts of the product. These values agreed well with those of DFA IV reported.[20] Therefore, the main product from levan was identified as DFA IV.

Cultural conditions for the DFA IV-producing LDE production

The effects of carbon sources of the LDE production medium on the DFA IV-producing LDE production were examined. As shown in Table II, carbon sources were added to the medium instead of levan at the same concentration. After cultivation for 24 h, the growth of this strain was good on all carbon sources except xylan, cellulose, and acetic acid. DFA IV-producing activity was detected only when levan was used as a carbon source. This enzyme was found to be induced specifically by levan, and only low levels of activities were detected with such carbon sources as fructose, xylose, lactose, and inulin. The effects of the concentration ratio of levan to yeast extract were also examined. The optimum concentrations were found to be 5 g/liter of levan and 0.5 g/liter of yeast extract (data not shown). Under these conditions, culture temperature raised from 27°C to 30°C to have better production of the LDE. Figure 1 shows the course of the LDE production by the strain GS-9. The growth reached maximum on 9 h incubation and the maximum DFA IV-producing activity of 3.3 units/ml was attained after incubation for 24 h.

Purification of the DFA IV-producing LDE

According to the methods described in Materials and Methods, the DFA IV-producing LDE was purified about 60-fold from the culture supernatant, as summarized in Table III. It gave a single band on SDS–PAGE as shown

Table II. Effects of Various Carbon Sources on DFA IV-Producing LDE Production

Carbon source	Growth (A_{600})	pH	Enzyme activity (units/ml)
Levan	9.5	7.7	2.4
Inulin	11.5	7.6	0.1
Xylan	0.8	8.1	0.0
Starch	10.5	7.4	0.0
Cellulose	0.0	8.0	0.0
Raffinose	11.6	7.6	0.0
Cellobiose	12.2	7.6	0.0
Lactose	10.8	7.3	0.1
Maltose	11.4	7.5	0.0
Xylose	13.4	7.6	0.1
Sucrose	11.3	7.5	0.0
Fructose	11.5	7.7	0.1
Glucose	10.7	7.6	0.0
Glycerol	15.0	7.4	0.0
Acetic acid	2.9	7.1	0.0
None	0.6	8.0	0.0

Cultivation was done as described under Materials and Methods for 24 h.

in Fig. 2A. The molecular weight of purified enzyme was estimated to be 52,000 by SDS–PAGE (Fig. 2A) and 48,000 by gel filtration with Bio-Gel P-150 (Fig. 2B). The enzyme from the strain GS-9 was considered to be a monomeric with a molecular weight of 52,000 and was different from the two enzymes from *A. ureafaciens*[9] and *Arthrobacter* sp. No. 11-E.[10]

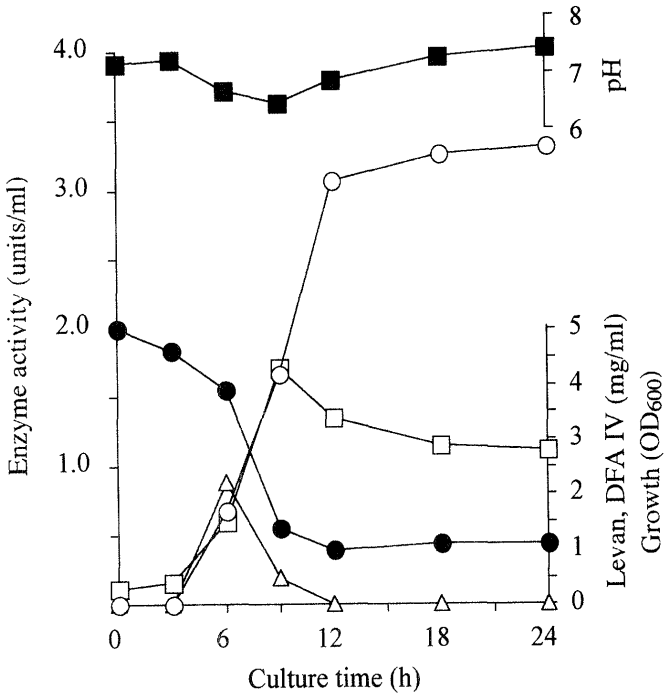

Fig. 1. Course of the DFA IV-Producing LDE Production by *Arthrobacter nicotinovorans* GS-9.

Cultures were done in the modified LDE production medium with 5 g/liter of levan and 0.5 g/liter of yeast extract at 30°C. ○, DFA IV-producing activity; □, growth; △, DFA IV; ●, total sugar; ■, pH.

pH and thermal properties

Effects of pH and temperature on both the reaction and stability of the purified LDE were examined. The activity was measured in 50 mM of Britton–Robinson buffer (pH 3.0 to 9.0). Maximal activity was obtained at pH 6.0. The effect of temperature on the enzyme activity was measured in the range of 20 to 70°C. The maximal activity was observed at 50°C. The purified enzyme was concentrated ten-fold with polyethylene glycol 20,000, and was mixed with 4 volumes of the 0.1 M of Britton-Robinson buffer (pH 3.0 to 12.0). After the incubation at 4°C for 24 h, the enzyme was diluted with 9 volumes of 0.1 M sodium phosphate buffer (pH 6.0) and was used as the treated enzyme. It was found that the enzyme was stable in the range of pH 4.0 to 12.0. The temperature stability was examined by heating the purified enzyme at various temperatures for 20 min. More than 90% of the enzyme activity remained up to 40°C.

Reaction products from levan

The reaction products from levan with purified enzyme were examined under the condition as follows. The reaction was done in a reaction mixture containing 50 mM of sodium phosphate buffer (pH 6.0), levan (10 mg/ml), and the enzyme (1.5 units/ml) in a total volume of 0.5 ml. The mixture was

Table III. Purification Steps of the DFA IV-Producing LDE from *Arthrobacter nicotinovorans* GS-9

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Culture supernatant	1697	648.6	2.6	100
$(NH_4)_2SO_4$ (60%)	521	62.8	8.3	30.7
DEAE-Toyopearl 650M	403	5.2	77.6	23.7
Bio-Gel P-100	110	1.0	113.7	6.5

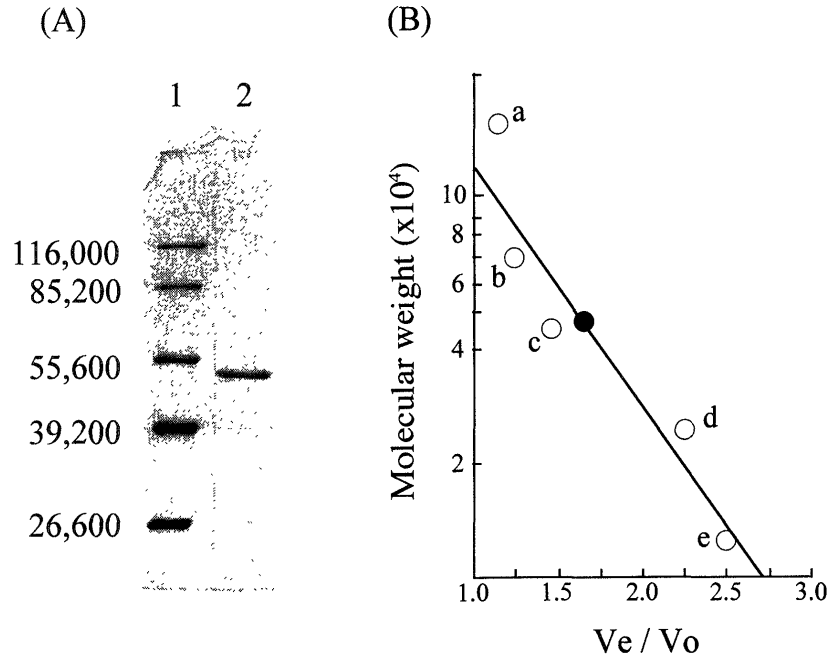

Fig. 2. SDS–PAGE (A) and the Gel Filtration (B) for Estimation of Molecular Weight of the Purified Enzyme.

(A): The gel was stained with silver stain. Lane 1, marker proteins; lane 2, purified enzyme. Standard maker proteins were β-galactosidase (M_r, 116,000), fructose-6-phosphate kinase (M_r, 85,200), glutamate dehydrogenase (M_r, 55,600), aldolase (M_r, 39,200), and triosephosphate isomerase (M_r, 26,600). (B): Plots of the logarithmic molecular weight *vs. Ve/Vo* in gel filtration with Bio-Gel P-150 were presented. The elution position of the sample enzyme is shown by a closed circle. a, aldolase (M_r, 158,000); b, bovine serum albumin (M_r, 68,000); c, egg albumin (M_r, 45,000); d, chymotrypsinogen *A* (M_r, 25,000); e, cytochrome *c* (M_r, 12,500).

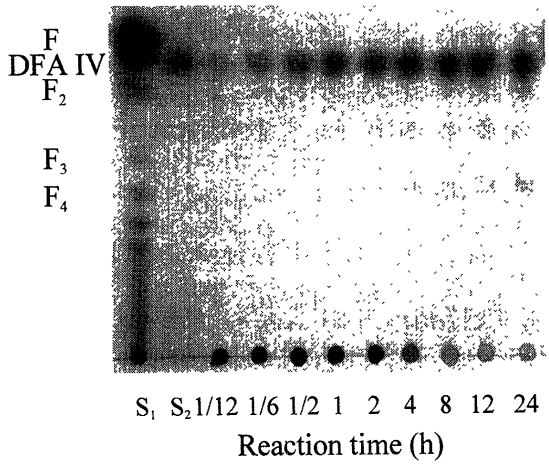

Fig. 3. TLC Analysis of the Reaction Products from Levan with Levan Fructotransferase.

The enzyme reaction was done as described in the text. S_1 was partial-HCl hydrolysates of levan. S_2 was purified DFA IV. F, fructose; F_2, levanbiose; F_3, levantriose; F_4, levantetraose.

Table IV. Reaction of the Levan Fructotransferase of *A. nicotinovorans* GS-9 on the Various Carbohydrates

Carbohydrate	Reaction[a] (%)[b]	Carbohydrate	Reaction
Levan	+ (75)	Fructose	−
LF_3	+ (18)	Glucose	−
LF_4	+ (37)	Melibiose	−
LF_5	+ (39)	Lactose	−
LF_6	+ (29)	Maltose	−
LF_7	+ (53)	Cellobiose	−
LF_2	−	Raffinose	−
IF_2	−	Melezitose	−
DFA IV	−	Stachyose	−
DFA III	−	Inulin	−
Sucrose	−	Starch	−
GF_2	−	Dextrin	−
GF_3	−	Xylan	−
GF_4	−	Mannan	−

LF_x, levanoligosaccharide; IF_x, inulooligosaccharide; GF_x, fructooligosaccharide.

[a] +, degraded; −, not degraded.

[b] Data in parenthesis are yield of DFA IV by wt% based on the initial levan.

incubated at 30°C for various times, and then heated in boiling water for 5 min to stop the enzyme reaction. As shown in Fig. 3, from the beginning of the reaction, DFA IV was observed as a main product which reached maximum (7.5 mg/ml) after the incubation for 24 h. Fructose, levanbiose, and two unidentified oligosaccharides were detected as minor products. Thus, the purified enzyme was considered to exogenously degrade levan and to produce DFA IV by intramolecular transfructosylation. The DFA IV-producing enzyme from the strain GS-9 was identified as a levan fructotransferase. As described above, the levan fructotransferase in this study was a monomer of molecular weight 52,000 and different from the levan fructotransferase of *A. ureafaciens*, which was reported as a dimer of homo subunits with a molecular weight of 60,000.[9] Therefore, the DFA IV-producing enzyme from *A. nicotinovorans* GS-9 was a novel levan fructotransferase.

Two unidentified oligosaccharides were estimated to be triose and tetraose, respectively, from the mobilities on TLC. They were supposed to have structures originated from levan side chains. The possible structures of these oligosaccharides were proposed to be β-2,1′-fructosyl-DFA IV[21] and β-2,1′-levanbiosyl-DFA IV, respectively. Identifications of these oligosaccharides are under study.

Substrate specificity of levan fructotransferase

The substrate specificity of the enzyme is shown in Table IV. The substrates were added to the reaction mixture instead of levan at the same concentration and reaction products incubated for 24 h were analyzed by TLC and HPLC. Under this condition, this enzyme reacted on levan and levanoligosaccharides having β-2,6-fructosyl linkages and whose degrees of polymerization are more than three, and gave DFA IV, levanbiose, and fructose as the products.

There are two possible mechanisms to produce DFA IV from levanoligosaccharides by levan fructotransferase in this study. One is the direct DFA IV formation by intramolecular transfructosylation, and another is DFA IV formation from elongated intermediate levanoligosaccharide by intermolecular fructosyl- and levanbiosyltransfer activity of levan fructotransferase.[22] Details of the second type of DFA IV formation are in progress and will be published elsewhere.

It has been shown that the yields of DFA IV increased with the increased degrees of polymerization. Thus, levan which has the high degrees of polymerization and low branching at the C-1 position is favorable for the high yield production of DFA IV. Consequently, levan produced by *Serratia levanicum*[5,12] is the most suitable substrate for DFA IV production by this enzyme.

Acknowledgments. The authors are grateful to Dr. K. Ochiai of Tokyo Research Laboratories of Kyowa Hakko Kogyo Co., Ltd. for two-dimensional electrophoresis of ribosomal proteins of the strain GS-9. The authors wish to thank GC-NMR Laboratory, Faculty of Agriculture, Hokkaido University for FD-MS and NMR spectroscopies.

References

1) S. Hestrin, S. Avineri-Shapiro, and M. Aschner, *Biochem. J.*, **37**, 450–456 (1943).
2) A. Yokota, S. Hirayama, K. Enomoto, Y. Miura, S. Takao, and F. Tomita, *J. Ferment. Bioeng.*, **72**, 258–261 (1991).
3) A. Yokota, K. Enomoto, and F. Tomita, *J. Ferment. Bioeng.*, **72**, 262–265 (1991).
4) A. Yokota, O. Yamauchi, and F. Tomita, *Lett. Appl. Microbiol.*, **21**, 330–333 (1995).
5) A. Yokota, K. Kondo, M. Nakagawa, I. Kojima, and F. Tomita, *Biosci. Biotech. Biochem.*, **57**, 745–749 (1993).
6) G. Avigad and S. Bauer, *Methods Enzymol.*, **8**, 621–628 (1966).
7) G. Avigad and R. Zelikson, *Bull. Res. Counc. of Israel*, **11A4**, 253–257 (1963).
8) K. Tanaka, T. Karigane, F. Yamaguchi, S. Nishikawa, and N. Yoshida, *J. Biochem.*, **94**, 1569–1578 (1983).
9) K. Tanaka, H. Kawaguchi, K. Ohno, and K. Shohji, *J. Biochem.*, **90**, 1545–1548 (1981).
10) H. Murakami, H. Muroi, H. Nakano, and S. Kitahata, *Kagaku to Kogyo* (in Japanese), **67**, 365–370 (1993).
11) A. Yokota and F. Tomita, *Nippon Jozokyokai Shi* (in Japanese), **89**, 626–633 (1994).
12) I. Kojima, T. Saito, M. Iizuka, N. Minamiura, and S. Ono, *J. Ferment. Bioeng.*, **75**, 9–12 (1993).
13) H. Sakurai, A. Yokota, Y. Sumita, Y. Mori, H. Matsui, and F. Tomita, *Biosci. Biotech. Biochem.*, **61**, 989–993 (1997).
14) M. Somogyi, *J. Biol. Chem.*, **195**, 19–23 (1952).

15) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350–356 (1956).
16) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265–275 (1951).
17) U. K. Laemmli, *Nature*, **227**, 680–685 (1970).
18) Y. Kodama, H. Yamamoto, N. Amano, and T. Amachi, *Int. J. Syst. Bacteriol.*, **42**, 234–239 (1992).
19) K. Ochiai and I. Kawamoto, *Biosci. Biotech. Biochem.*, **59**, 1679–1687 (1995).
20) K. Tanaka, T. Uchiyama, K. Yamauchi, Y. Suzuki, and S. Hashiguchi, *Carbohydr. Res.*, **99**, 197–204 (1982).
21) K. Tanaka, *Agric. Biol. Chem.*, **53**, 2275–2276 (1989).
22) K. Tanaka, T. Karigane, S. Fujii, T. Chinzaka, and S. Nagamura, *J. Biochem.*, **97**, 1679–1688 (1985).