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Purification of a Marine Bacterial Glucose Dehydrogenase from *Cytophaga marinoflava* and its Application for Measurement of 1,5-Anhydro-D-Glucitol

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ABSTRACT

A novel glucose dehydrogenase (GDH) from a marine bacterium *Cytophaga marinoflava* IFO 14170 was isolated from its membrane fraction. This GDH catalyzes the oxidation of a hydroxy group of glucose, but does not react in its C-1 position. This enzyme is composed of a single peptide with a mol wt of 67,000. The GDH can react under high salinity. The optimum pH is around 8.0, showing a typical property of marine bacterial enzymes. Using this novel enzyme, an enzymatic determination of 1,5-anhydro-D-glucitol (1,5AG) utilizing 2,6-dichrolophenolindophenol (DCIP) and phenazine methosulfate (PMS) as electron mediators was caried out. A good linear correlation was observed from 0.5 m*M* to 4 m*M* of 1,5AG.

Index Entries: Glucose dehydrogenase; Marine bacteria; 1,5-Anhydro-D-glucitol; diabetes; *Cytophaga marinoflava*.

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INTRODUCTION

Various glucose oxidoreductases are known and utilized for measurement of blood glucose level (*1*). β-D-glucose:oxygen 1-oxidoreductase (glucose oxidase [GOD]) (EC 1.1.3.4) is one of the most common enzymes for diagnosis. GOD has been extensively utilized for the enzyme sensor component for glucose measurement. Glucose dehydrogenases such as β-D-glucose:NAD (P)$^+$ 1-oxidoreductase (glucose dehydrogenase:(NAD[P]$^+$) (EC 1.1.1.47) and D-glucose:(acceptor) 1-oxidoreductase (glucose dehydrogenase:pyrroloquinoline quinone, PQQGDH) (EC 1.1.99.17) have also been used as components of enzyme-based biosensors. Among them, cofactor binding type GDHs are particularly useful because they are not affected by dissolved oxygen and do not need any additional expensive cofactor for the measurement.

Most of glucose oxidoreductases, except for a few enzymes, are 1-oxidoreductases that oxidize the 1st hydroxy group of glucose. Pyranose oxidase (EC 1.1.3.10) from basidiomycetes can react with the second hydroxy group of hexose (*2–5*) and GDH from *Agrobacterium tumefaciens* can react at the third position of glucose (3-GDH; EC 1.1.99.13) (*6,7*). These enzymes can be utilized in two ways.

First is the application of such enzymes for the bioconversion of sugars and their derivatives (*8,9*). The microbial conversion of disaccharides and their derivatives are of major interest for carbohydrate chemistry and technology. Disaccharides are available in very high purity and for a reasonable price; however, they are not yet being used as chemical intermediates. Since they have polyfunctional hydroxy groups, site-selective reactions are very difficult. Using *A. tumefaciens* derived 3-GDH, site-selective oxidation of such hydroxy group has been achieved. In contrast to sucrose, the 3-keto-derivatives, the products by 3-GDH, have at least one specific site for selective reaction. These disaccharide derivatives are expected to become starting material with novel commercial interest.

Second is the application of such enzymes for the measurement of hexoses that lack the first hydroxy group, for example, the measurement of 1,5-anhydro-D-glucitol (1,5AG).

1,5AG has a pyranoid structure, resulting from the deletion of an oxygen from glucose at the anomeric hydroxy group. Recently, the measurement of 1,5AG has been paid increasing attention to because 1,5AG in blood is known to decrease in diabetics (*10–12*). 1,5AG is not a substrate of glucose 1-oxidoreductase because it lacks a hydroxy group at C1 position.

In this study, we report the isolation of a novel GDH from the membrane fraction of the marine bacterium, *Cytophaga marinoflava*. Since this enzyme can oxidize 1,5AG, a possible use for this enzyme in 1,5AG assay is also presented.

MATERIALS AND METHODS

Chemicals

Polypeptone (Nihon Seiyaku, Tokyo, Japan), yeast extract (Difco, Detroit, MI), 1,5-anhydroD-glucitol (Wako Pure Chem., Osaka, Japan), α-methyl-D-glucoside (Kanto Chem., Tokyo, Japan), and 3-*o*-methylglucose (Aldrich, Milwaukee, WI) were purchased. All other chemicals were reagent grade.

Bacteria and Cultivation

A marine bacterium *C. marinoflava* IFO14170 (ATCC 19326) was used as the source of GDH. *C. marinoflava* was collected from an agar slant and inoculated into a subculture which was shaken at 25°C for 12–48 h in an L-tube containing a 5-mL medium consisting of 10 g polypeptone, 2 g yeast extract, 25 g NaCl, and 1 g $MgSO_4$ per 1000 mL of distilled water (pH 7.0). The subculture was inoculated into a 500-mL shaking flask containing 200 mL of medium. It was then incubated for 48 h at 30°C with rotary shaking. The cells harvested by centrifugation at 7000*g* at 4°C were resuspended in a 10-m*M* K-phosphate buffer containing 3% NaCl (pH 6.0). By repeating the centrifugation and suspension process, the cells were thoroughly washed. About 10 g (wet weight) of cells can be harvested from 1 L of culture.

Purification of Enzyme

Solubilization of the Enzyme

The membrane fraction was prepared from late log phase cells by passage through a French pressure cell (1500 kgf, three times) following removal of cell debris by centrifugation at 4000*g*, 20 min at 4°C, and collected by ultracentrifugation (69,800*g*, 90 min at 4°C) as pellets. From 20 g of wet cells, 2 g of membrane fraction could be collected. The membrane fraction was resuspended in a 10-m*M* K-phosphate buffer (pH 6.0) supplemented with Triton X-100 at a final concentration of 0.2% and stirred vigorously at 4°C for overnight. Then the solubilized membrane fraction was obtained as the supernatant after the ultracentrifugation (69, 800*g*, 90 min at 4°C).

The solubilized membrane fraction was then deionized by passing through a gel filtration column PD-10 (Pharmacia, Uppsala, Sweden), which consists of Sephadex G-25, or dialyzed overnight at 4°C against a 10-m*M* K-phosphate buffer (pH 5.5) containing 0.2% Triton X-100.

DEAE-Toyopearl Column Chromatography

The dialyzed or deionized enzyme preparation was applied to a DEAE-Toyopearl column (22 mm ID × 20 cm) (Tosoh, Tokyo, Japan) equilibrated with a 10-m*M* K-phosphate buffer (pH 5.5) containing 0.2% Triton X-100. After the column was washed with the same buffer, step gradient by every 10% of 0.75*M* NaCl 10-m*M* K-phosphate buffer (pH 5.5) was performed. The GDH active fraction was eluted at 0.15*M* of NaCl.

Hydroxyapatite Column Chromatography

After dialysis, the enzyme preparation was applied to a hydroxyapatite column (7.8 mm ID × 10 cm) (KB column; Koken, Tokyo, Japan) equilibrated with a 10-m*M* K-phosphate buffer (pH 6.0) containing 0.2% Triton X-100. After the column was washed with the same buffer, the enzyme was eluted with a linear gradient of 0–0.5*M* K-phosphate buffer (pH 6.0) supplemented with 0.2% Triton X-100.

Gel Filtration Chromatography

The enzyme solution from hydroxyapatite chromatography was dialyzed and applied to a TSKgel G3000 (8 mm ID × 30 cm) (Tosoh) equilibrated with a 10-m*M* K-phosphate buffer containing 0.3*M* NaCl and 0.2% Triton X-100 (pH 6.0). GDH positive fractions were collected and applied to the gel filtration chromatography again. GDH positive fractions were collected as purified enzyme sample.

SDS-PAGE

SDS-PAGE was performed in 8–25% polyacrylamide gradient gel (PhastGel gradient 8–25; Pharmacia, Sweden) using the Tris-tricine buffer system. The gels were stained for protein with silver nitrate. Separation and development were performed automatically by the Phast System (Pharmacia). The molecular mass was determined from the relative mobilities of standard proteins (a low molecular standard kit obtained from Pharmacia).

Assay of GDH Activity

Enzyme assay was carried out spectrophotometrically by measuring the reduction of the electron acceptor in accordance with the oxidation of glucose. The assay was performed using PMS coupled with DCIP as an electron acceptor. The reaction was carried out at 37°C in a polyethylene tube. Five microliters of enzyme solution were added into 20 μL of Tris-HCl buffer (pH 8.0, 25 m*M*) containing 0.75 m*M* of PMS and 0.75 m*M* of DCIP and preincubated for 1 min. The reaction was initiated by adding 1 μL of 2*M* glucose (final concentration of glucose was 80 m*M*) and incubated at 37°C. After 2 min, the sample was cooled by adding 100 μL of ice-cold distilled water, and the decrease in absorbance at 600 nm was measured using a spectrophotometer (UV 2200, Shimadzu, Kyoto, Japan)

equipped with ultra micro cell (100 μL). In the experiment of pH dependence, we used Na-phosphate buffer (pH 6–7.5) and glycine-NaOH buffer (pH 8.3–10.3) at 0.05 ionic strength.

Measurement of 1,5AG

Measurement of 1,5AG was carried out by the same method as described in "Assay of enzymatic activity." We used 1,5AG instead of glucose at a final concentration of 0–4 m*M* and incubated for 8 min.

RESULTS AND DISCUSSION

Purification of Enzyme

Screening of novel glucose dehydrogenases from various marine bacteria either isolated from Japanese coastal sea water or from culture collections was carried out. Among them, resting cells of the marine bacterium, *C. marinoflava* IFO 14170 (ATCC 19326; NCMB 397), was found to have unique GDH property showing activity toward α-methyl-D-glucoside, which normally was not a substrate of glucose 1-oxidoreductase. *C. marinoflava* was originally isolated by Colwell et al. in 1966 (*13*) as the host strain for bacteriophages. This marine bacterium is aerobic Gram-negative and can grow at 4–30°C, but cannot at 37°C. Disrupting and fractionating the cells, the GDH activity was concentrated only in the membrane fraction.

Enzyme purification was carried out as described in Materials and Methods. On SDS-PAGE, the purified enzyme migrated as a single species (Fig. 1), as judged by protein staining. The molecular mass of the purified enzyme was estimated at about 67,000. The enzyme probably consists of one subunit or homo subunits. Because of the highly hydrophobic property of this enzyme, mol-wt determination by gel filtration chromatography is, at the moment, impossible. This enzyme can utilize DCIP and PMS as the artificial electron mediator, but liberation of H_2O_2 from oxygen was not detected.

This enzyme was isolated in an active form, not requiring additional cofactors in order to show PMS-DCIP mediated GDH activity. The most well-known cofactor for the cofactor binding glucose dehydrogenase is pyrroloquinoline quinone (PQQ). In a number of Gram-negative bacteria, membrane-bound PQQ glucose dehydrogenases are found (*1*). Among them, PQQGDH of which PQQ can be easily removed by EDTA treatment are abundantly exist and are categorized as Type I PQQGDH (*14*).

Incubation of solubilized membrane fraction containing GDH activity in the presence of 100 m*M* EDTA for 10 min did not change PMS-DCIP mediated GDH activity (data not shown). This result indicates that this enzyme was not a Type I PQQGDH. Type II PQQGDHs of which activity

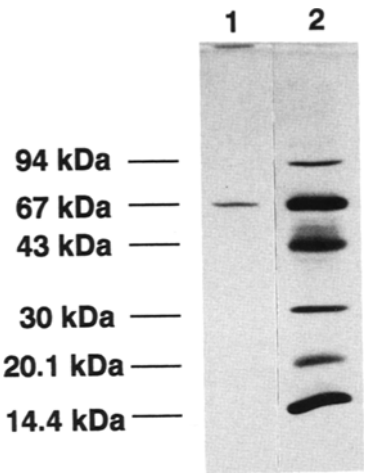

Fig. 1. SDS gel electrophoresis of glucose dehydrogenase. The conditions for SDS-PAGE are given under Materials and Methods. Lane 1, purified enzyme. Lane 2, standard proteins: phosphorylase b , 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsine inhibitor, 20. 1 kDa; α-lactalbumin, 14.4 kDa.

is not affected in the presence of EDTA are reported in *Gluconobacter suboxydans* (*15*) and *Acinetobacter calcoaceticus* (*14*). Usually, tightly bound PQQ in type II PQQGDHs will be liberated by heat treatment, and can be detected by bioassay of PQQ (*16*) utilizing *E. coli* derived apo-PQQGDH. Bioassay using cell free heat extract of *C. marinoflava* and *E. coli* derived apo-PQQDGH was carried out, however, PQQ was not detected. Considering that the purification procedure we achieved in this study was considerably low (purification fold; 33, recovery; 3.5%), optimization of purification procedure is necessary for further spectrophotometrical determination of the cofactor.

Although the cofactor of this enzyme remains unknown, these properties indicate the possibility of applying the enzyme as an oxygen insensitive assay.

Substrate Specificity of GDH

The substrate specificity of this enzyme is shown in Table 1. This enzyme can oxidize α-methyl-D-glucoside as well as glucose, although it lacks the first position of hydroxy group, indicating that this GDH is not a glucose 1-oxidoreductase. Furthermore, this enzyme can oxidize 2-deoxy-D-glucose (31%) and 1,5AG (48%), but not 3-*o*-methyl-D-glucose. The Km and Vmax for glucose are 5.5 m*M* and 2.9 U/mg protein and for 1,5AG are 6.6 m*M* and 1.8 U/mg protein.

This enzyme can oxidize 1,5AG; therefore, the enzymatic determination of 1,5AG utilizing this enzyme is possible. Considering that this

Table 1
Substrate Specificity of Glucose Dehydrogenase

Substrates		Relative activity, %
Aldohexose	D-Glucose	100
	Mannose	17
	D-Galactose	76
Aldopentose	D-Arabinose	8
	D-Xylose	27
	D-Ribose	2
Deoxy sugar	L-Rhamnose	3
	D-Fucose	4
	2-Deoxy-D-glucose	31
Ketohexose	L-Sorbose	10
	D-Fructose	4
Disaccharide	Maltose	33
	Sucrose	29
α-Methyl-D-glucoside		109
3-*o*-Methyl-glucoside		10
1,5-anhydro-D-glucitol		48

Enzyme assays were carried out spectrometrically at 37°C for 2 min. Various sugars (final concentration, 80 m*M*) were added to the reaction mixture as substrates.

enzyme scarcely reacts with 3-*o*-methyl-D-glucose but can oxidize other hydroxy group substituted sugars (1,5AG and 2-deoxy glucose), it is possible that this enzyme may oxidize the 3rd hydroxy group of glucose. Further study on the structure of produced derivatives is still required to define the site of the oxidation.

Optimization of Enzyme Reaction

In order to apply this enzyme for 1,5AG measurement, optimization of reaction condition was carried out. In a physiological pH and temperature range, pH or temperature profiles of enzyme reaction may reflect catalytic property and structural stability, but not the effect of conformational change of substrates. Since both glucose and 1,5AG will not change their equilibrium in conformational change within the physiological condition, the reaction condition will be examined by using glucose as a typical substrate, also considering its availability.

The effect of pH on the enzyme activity was examined from pH 6–10.3 (Fig. 2). The optimum pH was around 8.0 and this pH is almost the same as that of the sea water.

The effect of temperature on enzyme activity was also examined using glucose as the substrate (Fig. 3). The activity was measured at various

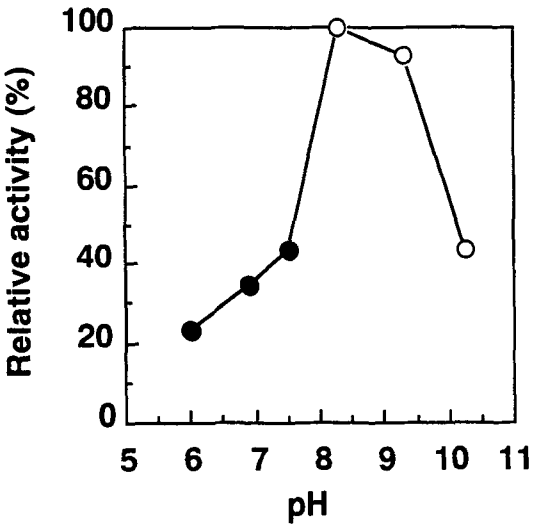

Fig. 2. Effect of pH on the activity of glucose dehydrogenase. The reactions were carried out for 2 min at 37°C in the following buffers with the ionic strength of 0.05: ●, Na-phosphate; ○, glycine-NaOH.

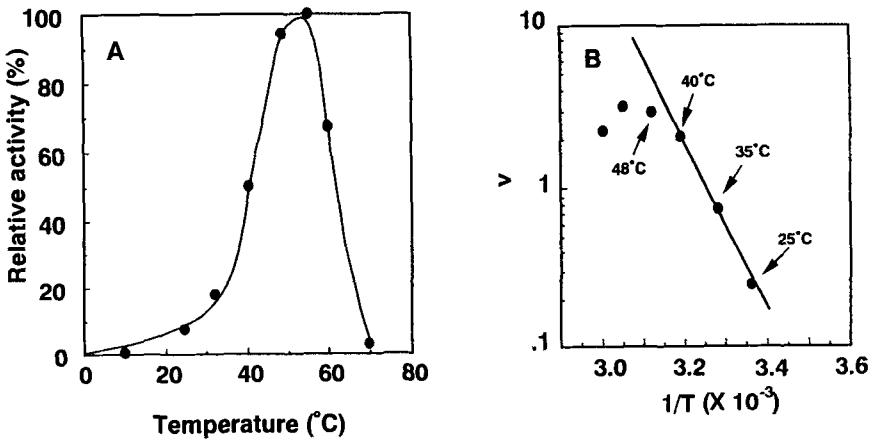

Fig. 3. Effects of temperature on the activity of glucose dehydrogenase. Relative activities at various temperatures (**A**) and Arrhenius plot (**B**). The reactions were carried out for 2 min at various temperatures.

temperatures over 10–70°C. The initial velocity of the reaction increased with the increase of temperature, reaching a maximum at 55°C (Fig. 3A). Figure 3B represents *Arrhenius* plot of the enzyme reaction. Temperatures were expressed as the reciprocal absolute temperature (K^{-1}), and the enzymatic activities were expressed as the reducing rate of DCIP (m*M*/min) at each temperature. A good linear correlation was observed at temperatures under 40°C. Up to 40°C, heat denaturation begins. Considering the optimum temperature of culture condition of *C. marinoflava* is 25°C, and cannot grow over 37°C, this enzyme might be not so stable

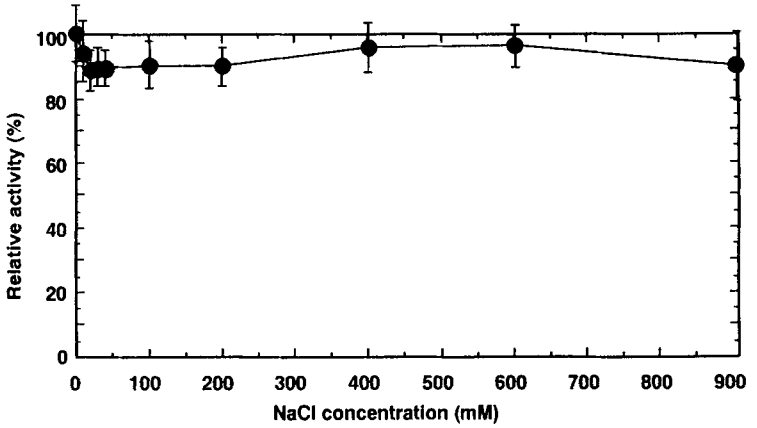

Fig. 4. Effect of NaCl concentration on the activity of glucose dehydrogenase. The reactions were carried out for 2 min in 25 m*M* Tris-HCl buffer (pH 8.0) containing various concentrations of NaCl.

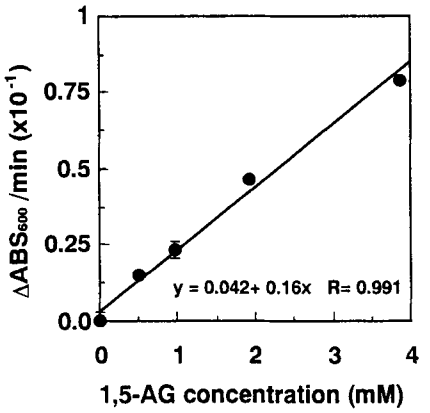

Fig. 5. Calibration curve of 1,5AG by spectrophotometry. The reactions were carried out for 8 min in 25 m*M* Tris-HCl (pH 8.0) at 37°C.

under high temperature. Therefore, in further experiment, the reaction was carried out at 37°C considering its thermal stability.

Since this enzyme is derived from marine bacterium, effect of NaCl on enzyme activity was also tested. Fig. 4 shows the relative activity at increasing levels of salinity, ranging from 0 to 900 m*M* NaCl compared to under 15 m*M* Tris-HCl (pH 8.0). From 0 to 20 m*M* of NaCl, the activity gradually reduced to 90% of initial value, but at a salinity higher than 20 m*M* GDH activity was not affected by the change in salinity. Even at a salinity of 900 m*M*, 90% of initial activity remained. This property suggests that this enzymatic reaction was not drastically affected by the change of salt concentration as was previously observed in PQQGDHs (*17*).

Spectrophotometric Measurement of 1,5AG

We investigated the measurement of 1,5AG. Figure 5 shows the correlation between authentic 1,5AG concentration and velocity of decrease

of absorbance at 600 nm, owing to the reduction of DCIP. A good linear correlation was observed between 1,5AG concentration and an optical-density decrease of DCIP solution, at 0.5–4 m*M* of 1,5AG. Since human plasma normally contains less than 0.1 m*M* 1,5AG, this assay method does not yet reach the level required for practical application. Further optimization of the mediator should lead to an improvement of the sensitivity of the assay.

In conclusion, here we report the isolation of a novel GDH which catalyzes oxidation of glucose probably at the C-3 hydroxy group. Utilizing the novel GDH, measurement of 1,5 AG was performed. Further application of this novel GDH for enzymatic conversion of disaccharides is being investigated.

REFERENCES

1. Fogarty, W. M. and Kelly, C. T. (1990), in *Microbial enzymes and Biotechnology*, 2nd ed., Elsevier, Essex, England, pp. 191–199.
2. Ruelius, H. W., Kervin, R. M., and Janssen, F. W. (1968), *Biochimica Biophysia Acta* **167,** 493–500.
3. Machida, Y. and Nakanishi, T. (1984), *Agric. Biol. Chem.* **48,** 2463–2470.
4. Volc, J., Denisova, N. P., Nerud, F., and Musilek, V. (1985), *Folia Microbiologica* **30,** 141.
5. Erikksson, K. E., Pettersson, B., Vol, J., and Musilek, V. (1986), *Applied Microbiol. Biotechnol.* **23,** 257.
6. Hayano, K. and Fukui, S. (1967), *J. Biol. Chem.* **242,** 3665–3672.
7. Van Beeumen, J. and De Ley, J. (1968), *Europ. J. Biochem.* **6,** 331–343.
8. Kunz, M. (1991), *Carbohydrates as Organic Raw Materials,* Lichtenthaler, F. W., ed., VCH, Weinheim, Germany, pp. 127–153.
9. Buchholz, K., Stoppok, E., Mantalla, K., Reh, K. D., and Joedening, H. J. (1991), *Carbohydrates as Organic Raw Materials,* Lichtenthaler, F. W., ed., VCH, Weinheim, Germany, pp. 155–168.
10. Servo, C. and Pitkänen, E. (1975), *Diabetologia* **11,** 575–580.
11. Akanuma, H., Ogawa, K., Lee, Y., and Akanuma, Y. (1981), *J. Biochem.* **90,** 157–162.
12. Yoshioka, S., Saitoh, S., Fujisawa, T., Fujimori, A., Takatani, O., and Funabashi, M. (1982), *Clin. Chem.* **28,** 1283–1286.
13. Colwell, R. R., Citarella, R. V., and Chen, P. K. (1966), *Can. J. Microbiol.* **12,** 1099–1103.
14. Dokter, P., Frank, J., and Duine, J. A. (1986), *Biochem. J.* **239,** 163–167.
15. Ameyama, M., Nonobe, M., Hayashi, M., Shinagawa, E., Matsushita, K., and Adachi, O. (1985), *Agric. Biol. Chem.* **49,** 1227–1231.
16. Ameyama, M., Nonobe, M., Shinagawa, E., Matsushita, K., and Adachi, O. (1985), *Analytical Biochem.* **151,** 263–267.
17. Sode, K., Matsumura, K., Tsugawa, W., and Tanaka, M. (1995), *J. Marine Biotechnol.* **2,** 214–218.