

Biohydrogen Production from Sucrose Using the Light-Harvesting Function of Zinc Chlorophyll-*a*

Yumi Takeuchi and Yutaka Amao*

Department of Applied Chemistry, Oita University, Dannoharu 700, Oita 870-1192

Received April 19, 2004; E-mail: amao@cc.oita-u.ac.jp

A biohydrogen production system, has been developed that couples sucrose hydrolysis by invertase and glucose dehydrogenase (GDH) and hydrogen production with a platinum colloid as a catalyst. The system uses the visible light-harvesting function of artificial Zn chlorophyll-*a* (Zn Chl-*a*), prepared from Mg Chl-*a* (obtained from *Spilurina*). When a sample solution containing sucrose, invertase, nicotinamide adenine dinucreotide (NAD^+), Zn Chl-*a*, methylviologen (MV^{2+}, an electron carrier), and platinum colloid was irradiated, continuous hydrogen production was observed with the irradiation time. The amount of hydrogen production was about 10.5 μmol after 4 h of irradiation under the optimum condition.

Biohydrogen production systems from bio-resources, such as starch, cellulose, sucrose, and lactose, are important for the environment and the development of energy sources.[1–5] These polysaccharides or oligosaccharides are hydrolyzed to form monosaccharides, such as glucose. The conversion of glucose to hydrogen will be a useful new enzymatic pathway. Some studies on hydrogen production from glucose using an enzymatic pathway have been reported.[6–10] Hydrogen production from glucose by combining glucose dehydrogenase (GDH) and hydrogenase has been reported.[11,12] Since glucose was obtained from sucrose by using invertase enzymatically, hydrogen production from an oligosaccharide, sucrose, will be attained using the combination of invertase, GDH and hydrogenase or the other hydrogen-evolved catalyst.

On the other hand, some photoinduced hydrogen production systems consist of an electron donor, a photosensitizer, an electron carrier, and a catalyst for hydrogen production.[13–19] For a hydrogen-evolved catalyst, platinum colloid[17–19] and hydrogenase from *Desulfovibrio vulgaris* (Miyazaki)[13–16] are widely used in hydrogen-production systems. Especially, platinum colloid is stable against long-term irradiation. Because water-soluble zinc porphyrins have an absorption band in the visible light region, these porphyrins have been widely used as effective photosensitizers in a photoinduced hydrogen-production system.[13,14] However, the molar absorption coefficient of zinc porphyrins in the visible light region (500–600 nm) is lower than that in the near ultra-visible light region (380–400 nm). In contrast, Mg chlorophyll-*a* (Mg Chl-*a*), which acts as the light-harvesting function in the photosynthesis of green plants, has an absorption maximum at 670 nm;[20] also, Mg Chl-*a* is an attractive compound as a visible photosensitizer. We previously reported on photoinduced hydrogen-production systems with Mg Chl-*a* and platinum colloid.[21–23] In general, purified Mg Chl-*a* is unstable against irradiation. Recently, zinc bacteriochlorophyll-*a* was found to exist in an aerobic bacterium, *Acidiphilium rubrum*.[24] Because the zinc porphyrins are stable against irradiation and are effective photosensitizers, zinc chlorophylls are also attractive compounds as stable visible photosensitizers. The preparation and characterization of zinc chlorophyll-*a* (Zn Chl-*a*) and bacteriochlorophylls have been reported.[20,25] We previously reported on the preparation and characterization of Zn Chl-*a*, and found that the photositability of Zn Chl-*a* was superior to that of Mg Chl-*a* under various pH conditions.[26,27]

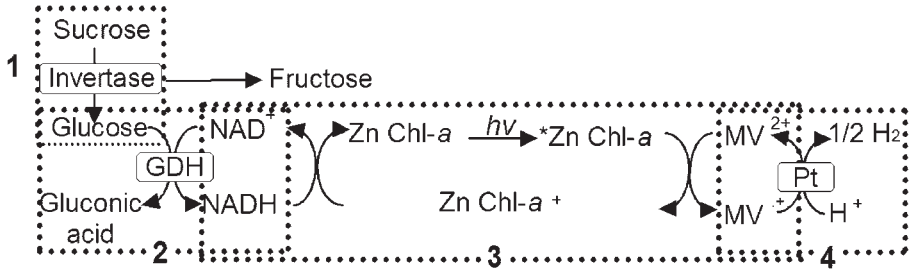
In photoinduced hydrogen production with a system consisting of an electron donor, a photosensitizer, an electron relay, and a catalyst, a photoexcited photosensitizer reacts with an electron relay to form a reduced electron relay; hydrogen evolves by proton reduction with the catalyst, and then the oxidized photosensitizer is reduced by an electron-donating reagent, such as reduced nicotinamide adenine dinucreotide (NADH). Thus, the electron donor, NADH, was a sacrificial reagent, and the oxidized electron donor, NAD^+, was consumed in the reaction system. If NADH is regenerated, a photoinduced hydrogen-production system is accomplished without NAD^+ consumption. Since GDH uses NAD^+ as a cofactor, photoinduced hydrogen production with GDH, an electron donor, a photosensitizer, an electron relay reagent, and a catalyst will be attained. An effective photoinduced hydrogen production system will be accomplished using the light-harvesting function of Zn Chl-*a* instead of Mg Chl-*a*.

In this paper we describe a biohydrogen production system coupling sucrose hydrolysis with invertase and GDH, and hydrogen production with a platinum colloid using the light-harvesting function of artificial Zn Chl-*a* in the presence of methylviologen (MV^{2+}) as an electron carrier, as shown in Scheme 1.

Experimental

Mg Chl-*a* from *spirulina*, invertase from Yeast and GDH from *Bacillus* sp. were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). NAD^+ and NADH were purchased from Oriental Yeast Co., Ltd. MV^{2+} dichloride and cetyltrimethylammonium bromide (CTAB) were punched from Tokyo Kasei Co., Lid

Published on the web April 8, 2005; DOI 10.1246/bcsj.78.622



Scheme 1. Biohydrogen production system coupling sucrose hydrolysis with invertase and GDH and hydrogen production with platinum colloid via MV^{2+} photoreduction using light-harvesting function of Zn Chl-*a*.

(Tokyo, Japan). Hydrogen hexachloplatinate hexahydrate and sodium citrate dihydrate were obtained from Kanto Chemical Co., Ltd (Tokyo, Japan). The other chemicals were of analytical grade or the highest grade available. One unit of GDH activity was defined as the amount of enzyme that reduced 1.0 μmol NAD^+ to NADH by glucose per min. One unit of invertase activity was defined as the amount of enzyme that produced 1.0 μmol of glucose by sucrose per min.

Zn Chl-*a* was prepared according to a previously reported method.[25,26] Zn Chl-*a* was synthesized by refluxing Mg Chl-*a* (50 mg, 56 μmol) with about a 10-times molar equivalent of zinc acetate in 100 mL of methanol at 80 °C for 5 h. The insertion of zinc ion into the porphyrin ring of Chl-*a* was monitored by the visible absorption spectrum. During the reaction, the characteristic absorption bands of Zn Chl-*a* at 421 and 662 nm increased, and the absorbance bands at 433 and 668 nm of Mg Chl-*a* gradually decreased. After the mixture was cooled to room temperature, the solvent was removed by using a rotary evaporator, and the reaction mixture was then washed with water to remove any unreacted zinc acetate. Finally, Zn Chl-*a* was precipitated in water. Zn Chl-*a* was collected by filtration and washed with water. The purification was performed by recrystallization (water–methanol). Zn Chl-*a* and Mg Chl-*a* were solubilized with 10 mmol dm^{-3} of CTAB, since Zn Chl-*a* and Mg Chl-*a* are insoluble to aqueous solution.

Platinum colloid was prepared by the reduction of a hexachloplatinate solution with sodium citrate. The reduction procedure was similar to a previously reported method.[17] A solution of 400 mL of water containing 30 mg of hydrogen hexachloplatinate hexahydrate was brought to boiling temperature using a mantle heater with a magnetic stirrer for 1.5 h, and then a solution of 30 mL of water containing 600 mg of sodium citrate dihydrate was added and refluxed with a magnetic stirrer at 100 °C for 4 h. The particle size of prepared platinum colloid was estimated to be 1.5 nm. The prepared platinum colloid had the ability to release 0.7 μmol of hydrogen in a reaction system including 10 μL of colloidal platinum, 1.2×10^{-5} mmol of MV^{2+} and 7.7×10^{-5} mmol of sodium dithionite in 4.0 mL of 50 mmol dm^{-3} Tris–HCl buffer (pH 7.4) at 30 °C for 10 min. One unit of colloidal platinum activity was defined as the release of 1.0 μmol of hydrogen per min.

The reduction of NAD^+ was tested in a reaction mixture containing NAD^+, sucrose, invertase, and GDH. The reaction was started by the addition of an NAD^+ solution to a sample solution containing sucrose (0.375 mmol dm^{-3}), invertase (4.0 units) and GDH (5.0 units) in 3.0 mL of 10 mmol dm^{-3} potassium phosphate buffer (pH 7.0) (process A and B in Scheme 1). The reduction of NAD^+ to NADH by GDH was determined by following the specific absorption at 340 nm, assuming a molar extinction coefficient of 6.3×10^3 $mol^{-1}\,dm^3\,cm^{-1}$. To investigate the effect of the NAD^+ concentration on NADH reduction, NAD^+ was changed from 1.75 to 5.25 mmol dm^{-3}.

The photoreduction of MV^{2+} was tested in a reaction mixture containing NAD^+, sucrose, Zn Chl-*a*, MV^{2+}, invertase, and GDH. The reaction system consisted of NAD^+, sucrose (100 mmol dm^{-3}), Zn Chl-*a* (15 μmol dm^{-3}), MV^{2+} (0.4 mmol dm^{-3}), invertase (4.0 units), and GDH (5.0 units) in 3.0 mL of 10 mmol dm^{-3} potassium phosphate buffer (pH 7.0). The sample solution was deaerated by repeated freeze–pump–thaw cycles, and substituted by argon gas. Then, the sample solution was irradiated with a 200 W tungsten lamp at a distance of 3.0 cm, with a light intensity of 200 J m^{-2} s^{-1}, at 30 °C. Light with wavelengths of less than 390 nm was removed by a Toshiba L-39 cut-off filter (Tokyo, Japan). The reduction of MV^{2+} was monitored using a UV–vis spectrophotometer at 605 nm, with a molar extinction coefficient of 1.3×10^4 $mol^{-1}\,dm^3\,cm^{-1}$.[28] To investigate the effect of the NAD^+ concentration on the MV^{2+} reduction, the NAD^+ concentration was changed from 1.75 to 5.25 mmol dm^{-3}.

Photoinduced hydrogen production from sucrose was carried out as follows. A sample solution containing NAD^+, sucrose (100 mmol dm^{-3}), Zn Chl-*a* (15 mmol dm^{-3}), MV^{2+} (0.4 mmol dm^{-3}), platinum colloid (0.5 unit), invertase (4.0 units), and GDH (5.0 units) in 3.0 mL of 10 mmol dm^{-3} potassium phosphate buffer (pH 7.0) was deaerated by a freeze–pump–thaw cycle 6 times, and substituted by argon gas. The amount of hydrogen evolved was measured by Schimadzu GC-14B gas chromatography (detector, TCD; column temperature, 40 °C; column, active charcoal with the particle size 60–80 mesh; carrier gas, nitrogen gas; carrier gas flow rate, 24 mL min^{-1}). To investigate the effect of the NAD^+ concentration on hydrogen production, the NAD^+ concentration was changed from 1.75 to 5.25 mmol dm^{-3}.

Results and Discussion

The time dependence of NADH formed in a solution containing sucrose, invertase, GDH, and NAD^+ is shown in Fig. 1. The initial rate of NADH formation was determined by the amount of NADH after incubation for 10 min. In all cases of NAD^+ concentrations, there was no difference in the initial rate of NADH formation. In contrast, no formation of NADH was observed in a solution containing sucrose, GDH, and NAD^+ (closed triangle). Thus, NADH was formed via sucrose hydrolysis with invertase. After 50 min of incubation, 0.375 mmol dm^{-3} NADH was formed. This value is equal to the initial concentration of sucrose. This result shows that sucrose was hydrolyze to glucose and fructose completely.

The time dependence of the $MV^{\bullet+}$ concentration in a system containing sucrose, invertase, GDH, NAD^+, Zn Chl-*a*,

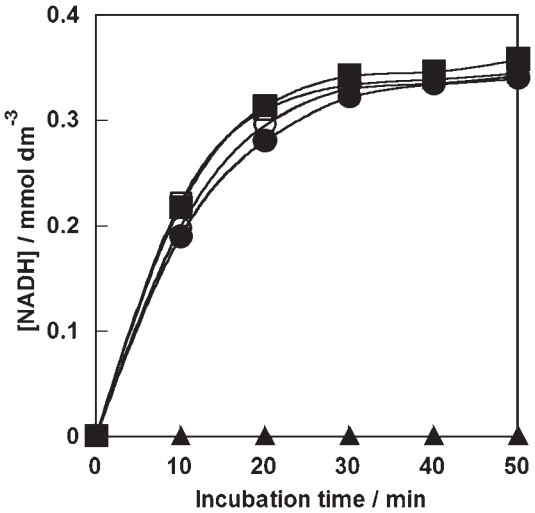


Fig. 1. Time dependence of NADH formation with sucrose (0.375 mmol dm^{-3}), invertase (4 units), NAD^+, and GDH (5 units) in in 3.0 mL of 10 mmol dm^{-3} phosphate buffer (pH 7.0). NAD^+ (open circle): 1.75, (closed square): 2.63, (open square): 3.5, and (closed circle): 5.25 mmol dm^{-3}. (closed triangle): without NAD^+.

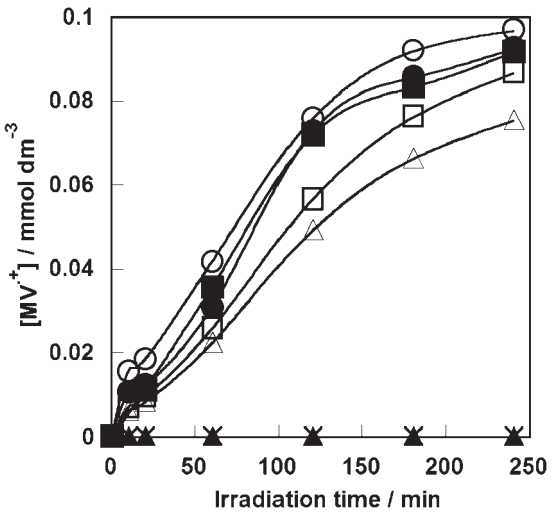


Fig. 2. The time dependence of the reduced MV^{2+} concentration under steady state irradiation with visible light using 200 W tungsten lamp at a distance of 3.0 cm. The sample solution consisting of NAD^+, sucrose (20 mmol dm^{-3}), invertase (4 units), GDH (5 units), Zn Chl-*a* (15 μmol dm^{-3}), and MV^{2+} (0.4 mmol dm^{-3}) in 3.0 mL of 10 mmol dm^{-3} potassium phosphate buffer (pH 7.0). NAD^+ (open circle): 1.75, (closed square): 2.63, (open square): 3.5, and (closed circle): 5.25 mmol dm^{-3}. (closed triangle): without NAD^+. (× symbol): without irradiation. Open triangle: 10 mmol dm^{-3} potassium phosphate buffer prepared using D_2O is used as the reaction media (pH 7.0).

and MV^{2+} with visible light irradiation is shown in Fig. 2. The absorbance at 605 nm, the absorption band of $MV^{\bullet+}$, increased with the irradiation time. In all cases of NAD^+ concentrations, ca. 0.1 mmol dm^{-3} reduced MV^{2+} is produced and the yield of

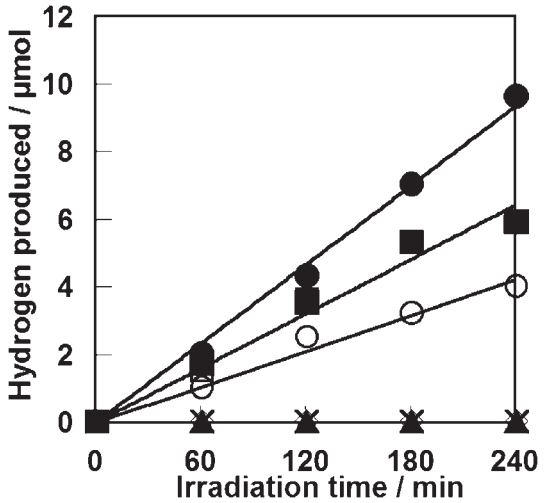


Fig. 3. Time dependence of hydrogen production under steady state irradiation with visible light using 200 W tungsten lamp at a distance of 3.0 cm. The sample solution consisting of NAD^+, sucrose (20 mmol dm^{-3}), invertase (4 units), GDH (5 units), Zn Chl-*a* (15 μmol dm^{-3}), MV^{2+} (0.4 mmol dm^{-3}), and platinum colloid (0.5 unit) in 3.0 mL of 10 mmol dm^{-3} potassium phosphate buffer (pH = 7.0). NAD^+ (open circle): 1.75, (closed square): 2.63, (open square): 3.5, and (closed circle): 5.25 mmol dm^{-3}. (closed triangle): without NAD^+. (× symbol): without irradiation. Open rhombic: 10 mmol dm^{-3} potassium phosphate buffer prepared using D_2O is used as the reaction media (pH 7.0).

MV^{2+} to $MV^{\bullet+}$ was estimated to be ca. 25% after 240 min of irradiation. The photoreduction rate was independent of the concentrations of NAD^+, sucrose, invertase, and GDH. In contrast, the reduction rate depended on the concentrations of Zn Chl-*a* and MV^{2+}. Thus, the rate-limiting step in the MV^{2+} reduction (processes 1, 2, and 3 in Scheme 1) was the electron-transfer process from the photoexcited Zn Chl-*a* (*Zn Chl-*a*) to MV^{2+} (process 3 in Scheme 1). In contrast, MV^{2+} was not reduced without NAD^+ in the above system (closed triangle), or without irradiation (× symbol). Thus, the MV^{2+} photoreduction proceeded by coupling the sucrose hydrolysis with invertase and GDH (processes 1 and 2 in Scheme 1) and MV^{2+} reduction using the light-harvesting function of Zn Chl-*a* (process 3 in Scheme 1).

Figure 3 shows the time dependence of visible light-induced hydrogen production in a system containing sucrose, invertase, GDH, NAD^+, Zn Chl-*a*, MV^{2+}, and platinum colloid. By irradiation, hydrogen was produced continuously for more than 4 h. In contrast to the MV^{2+} photoreduction, the hydrogen production depended on the NAD^+ concentration. This result indicates that the rate-limiting step in the photoinduced hydrogen production of Scheme 1 was the electron-transfer process from the NADH to one-electron oxidized Zn Chl-*a* (Zn Chl-a^+). By adding platinum colloid to the MV^{2+} photoreduction (1, 2, and 3 in Scheme 1), the reaction of $MV^{\bullet+}$ to MV^{2+}, due to hydrogen production with platinum colloid (process 4 in Scheme 1), proceeded rapidly. On the other hand, NADH formation (process 1 and 2 in Scheme 1) also proceeded rap-

idly, as shown in Fig. 1. The amount of hydrogen produced increased with increasing the NAD^+ concentration, indicating that the rate-limiting step was the electron-transfer process from the NADH, produced with process 1 and 2 in Scheme 1, to Zn Chl-a^+. Thus, the hydrogen production depended on the NAD^+ concentration. The amount of hydrogen production was estimated to be 10.5 μmol after 4 h of irradiation (closed circle) under 5.25 mmol dm^{-3} NAD^+. In contrast, the hydrogen production was 4.0 μmol after 4 h of irradiation (open circle) under 1.75 mmol dm^{-3} NAD^+. The turnover number of Zn Chl-*a* was estimated to be 442 h^{-1} under 5.25 mmol dm^{-3} NAD^+. Therefore, the Zn Chl-*a* appears to have served as a system for transferring electrons from NADH, which was formed from sucrose, to a more reductive hydrogen molecule. On the other hand, hydrogen also was not evolved in the absence of NAD^+ in the above system (closed triangle) or without irradiation (× symbol). These results strongly suggest that visible light-induced hydrogen production proceeded by coupling the sucrose hydrolysis with invertase and GDH (processes 1 and 2 in Scheme 1), and that hydrogen production with platinum colloid proceeding by using the light-harvesting function of Zn Chl-*a* (processes 3 and 4 in Scheme 1).

Next, to investigate the proton source of photoinduced hydrogen production in a system containing sucrose, invertase, GDH, NAD^+, Zn Chl-*a*, MV^{2+}, and platinum colloid, 10 mmol dm^{-3} potassium phosphate buffer prepared using D_2O was used as the reaction media (pH 7.0). At first, the photoreduction of MV^{2+} was attempted in 10 mmol dm^{-3} potassium phosphate buffer prepared using D_2O. The time dependence of the $MV^{\bullet+}$ concentration in a system containing sucrose, invertase, GDH, NAD^+, Zn Chl-*a*, and MV^{2+} in 10 mmol dm^{-3} potassium phosphate buffer prepared using D_2O with visible light irradiation is shown in Fig. 2 (open triangle). The photoreduction of MV^{2+} proceeded in a potassium phosphate buffer prepared using D_2O. However, no hydrogen production with a system containing sucrose, invertase, GDH, NAD^+, Zn Chl-*a*, MV^{2+}, and platinum colloid in 10 mmol dm^{-3} potassium phosphate buffer, prepared using D_2O, was observed, as shown in Fig. 3 (open rhombic). This result shows that the platinum colloid could not catalyze the reduction of D^+ to D_2. Thus, the proton source of the photoinduced hydrogen production was the proton included into reaction media. The possible reason why the no hydrogen production occurred in a potassium phosphate buffer prepared using D_2O is the difference between D_2O and H_2O in pK_a value, the redox potential, and so on. However, the reason has not yet been clarified, and is now under research.

In conclusion, a hydrogen-production system coupling sucrose hydrolysis with invertase and GDH and hydrogen production with platinum colloid via MV^{2+} photoreduction using the light-harvesting function of Zn Chl-*a* has been developed and continuous hydrogen gas achieved. Only gluconic acid is produced as a by-product, and no carbon dioxide gas is evolved in present reaction system. Thus, renewable bio-resources have been effectively used to produce hydrogen gas through an environmentally clean process.

This work was partially supported by the Foundation for Promotion of Material Science and Technology of Japan, The Japan Securities Scholarship Foundation and a Grant-in-Aid for Scientific Research Areas (417) from the Ministry of Education, Culture, Sports, Science and Technology.

References

1 L. C. Helena and R. P. Overend, *Fuel Process. Technol.*, **71**, 187 (2001).
2 M. J. Barbosa, J. M. Rocha, J. Tramper, and R. H. Wijffels, *J. Biotechnol.*, **85**, 25 (2001).
3 L. Garcia, R. French, S. Czernik, and E. Chornet, *Appl. Catal., A*, **201**, 225 (2000).
4 D. Wang, *Fuel Energy Abstr.*, **39**, 188 (1998).
5 T. Minowa and S. Inoue, *Renewable Energy*, **16**, 1114 (1999).
6 V. R. Rustamov, K. M. Abdullayev, F. G. Aliyev, and V. K. Kerimov, *Int. J. Hydrogen Energy*, **23**, 649 (1998).
7 J. Woodward, M. Orr, K. Cordray, and E. Greenbaum, *Nature*, **405**, 1014 (2000).
8 O. Mizuno, R. Dinsdale, F. R. Hawkes, D. L. Hawkes, and T. Noike, *Bioresour. Technol.*, **73**, 59 (2000).
9 M. Akashah, M. Yoshida, M. Watanabe, M. Nakamura, and J. Matsumoto, *Water Sci. Technol.*, **36**, 279 (1997).
10 K. Ma and M. W. Adams, *J. Bacteriol.*, **176**, 6509 (1994).
11 J. Woodard, S. M. Mattingly, M. Danson, D. Hough, N. Ward, and M. Adams, *Nat. Biotechnol.*, **14**, 872 (1996).
12 T. Inoue, S. N. Kumar, T. Kamachi, and I. Okura, *Chem. Lett.*, **1999**, 147.
13 I. Okura, *Coord. Chem. Rev.*, **68**, 53 (1985).
14 I. Okura, *Biochimie*, **68**, 189 (1986).
15 I. Okura, S. Aono, and S. Kusunoki, *Inorg. Chim. Acta*, **71**, 77 (1983).
16 I. Okura and N. Kim-Thuan, *J. Chem. Soc., Faraday Trans. 1*, **77**, 1411 (1981).
17 J. R. Darwent, P. Douglas, A. Harriman, G. Porter, and M. C. Richoux, *Coord. Chem. Rev.*, **44**, 93 (1982).
18 P. A. Brugger, P. Cuendet, and M. Grätzel, *J. Am. Chem. Soc.*, **103**, 2923 (1981).
19 J. Kiwi and M. Grätzel, *J. Am. Chem. Soc.*, **101**, 7214 (1979).
20 H. Scheer, "Chlorophylls," CRC Press, London (1991).
21 Y. Tomonou and Y. Amao, *BioMetals*, **15**, 391 (2002).
22 Y. Tomonou and Y. Amao, *BioMetals*, **16**, 419 (2003).
23 N. Himeshima and Y. Amao, *Biotechnol. Lett.*, **24**, 1935 (2002).
24 M. Kobayashi, M. Yamamura, M. Akiyama, H. Kise, K. Inoue, M. Hara, N. Wakao, K. Yahara, and T. Watanabe, *Anal. Sci.*, **14**, 1149 (1998).
25 N. Wakao, N. Yokoi, N. Isoyama, A. Hiraishi, K. Shimada, M. Kobayashi, H. Kise, M. Iwaki, S. Itoh, S. Takaichi, and Y. Sakurai, *Plant Cell Physiol.*, **37**, 889 (1996).
26 Y. Takeuchi and Y. Amao, *BioMetals*, **18**, 15 (2005).
27 Y. Takeuchi and Y. Amao, *Bioconjugate Chem.*, **14**, 268 (2003).
28 T. Watanabe and K. Honda, *J. Phys. Chem.*, **86**, 2617 (1982).