Anal. Chem. **2009,** *81,* 9383–9387

Coulometric D-Fructose Biosensor Based on Direct Electron Transfer Using D-Fructose Dehydrogenase

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This paper describes a batch-type coulometric D-fructose biosensor based on direct electron transfer reaction of D-fructose dehydrogenase (FDH) adsorbed on a porous carbon electrode surface. The adsorbed-FDH electrodes catalyzed the electrochemical two-electron oxidation of D-fructose to 5-keto-D-fructose without a mediator. Nanostructured carbon particle-modified electrodes were used for the coulometric D-fructose biosensor to enhance the catalytic current density. The electric charge for the D-fructose oxidation gained by the biocoulometric measurement was in good agreement with the theoretical value corresponding to D-fructose amount in the range from 1 to 100 mM with a sample volume of 1 μL. This method is also applicable to the determination of several oligo/polysaccharides containing the D-fructose unit, in combination with specific hydrolases to yield D-fructose. An example was demonstrated by sucrose determination in which the electrode modified with FDH and invertase was used as a working electrode. To address the problem of electroactive interferences such as ascorbate, the electric charge at the FDH-free electrode was subtracted from the total charge obtained at the FDH-adsorbed electrode. The D-fructose concentrations in several beverages were successfully determined with this method.

Electrochemical biosensors based on bioelectrocatalytic reaction are simple devices that convert enzymatic reactions into electrochemical signals, with the advantage of rapid and accurate measurements. Amperometry and coulometry are the two types of electrochemical biosensing methods.[1,2] Most commercial biosensors employ amperometric determination because of its fast response. However, amperometric technology measures only a limited part of the sample, and sample readings may be strongly affected by the kinetics of the enzymatic reaction, the mass transfer in the catalytic layer, etc. Thus, it requires a calibration procedure to convert currents to concentration values under strictly controlled conditions. In addition, a weak signal being generated is particularly noticeable at low substrate concentrations. In contrast, coulometry is a method of absolute quantitative analysis, converting all the measured substance into electric charge and thus inherently needs no calibration procedure. In principle, there are no upper and lower theoretical detection limits as long as the samples are completely electrolyzed and a wide concentration range of determination is possible. Moreover, the total charge in complete electrolysis is not affected by enzymatic activity and mass-transfer processes. Heller has developed Os complex-mediated coulometric sensing system for a blood glucose sensor.[1] Abbott/TheraSense provided the blood glucose monitor FreeStyle, in which a thin-layer microcoulometric system allowed the measurement of the blood-glucose concentration in a blood sample as small as 300 nL.

In this work, we developed a coulometric electrochemical biosensor of D-fructose based on direct electron transfer (DET)-type reaction. Determination of D-fructose is expected to be utilized in the field of clinical and food analyses.[3−6] Reliable D-fructose biosensors would be in great demand to detect D-fructose not only in food products but also in clinical samples. D-Fructose oxidation to 5-keto-D-fructose can be enzymatically catalyzed by D-fructose dehydrogenase (FDH) from *Gluconobacter frateurii*.[8,9] As FDH shows high substrate specificity to D-fructose and oxygen is completely inactive as an electron acceptor, this enzyme is expected to be suitable for electroenzymatic determination of D-fructose. When FDH is adsorbed onto the electrode, it can transfer electrons from D-fructose to the electrode directly. Several amperometric biosensors based on DET have been reported.[9−11] The advantage of DET-based biosensors is that the lack of mediators simplifies the reaction system, which will make a significant impact on the sensor fabrication. However, coulometric biosensors based on DET have not yet been reported. The reason why most of the researchers have avoided coulometric biosensors based on DET lies in the long time required to complete the electrolysis reaction as a consequence of the low current density with DET-based reactions. However, we have reported recently that the current density for the electrochemical oxidation of D-fructose catalyzed by FDH was significantly improved by using

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10.1021/ac901771t CCC: $40.75
Published on Web 10/16/2009

porous carbon electrode.[12,13] Such enhancement of the DET-based bioelectrocatalytic current densities enabled the realization of the coulometric D-fructose biosensor described here. The amount of sucrose was also determined with this coulometric biosensor by using the carbon black-modified carbon paper electrode in combination with FDH and invertase. Last, we hereby suggest a simple method to eliminate the effect of electroactive interfering compounds contained in real samples without the need of special membranes: the electric charge at the FDH-free electrode is subtracted from the total charge obtained at the FDH-adsorbed electrode.

EXPERIMENTAL SECTION

Enzymes and Reagents. FDH (EC 1.1.99.11, from *Gluconobacter frateurii*) and invertase (EC 3.1.2.26, from *Saccharomyces cerevisiae*) were purchased from Toyobo Co., Ltd., and Sigma-Aldrich, Inc., respectively, and used without further purifications. The concentration of the FDH stock solution was determined spectrophotometrically using a molar extinction coefficient of the heme c groups ($\varepsilon_{550\ nm} = 23\ 000\ M^{-1}\ cm^{-1}$).[14] D-Fructose and sucrose were purchased from Wako Pure Chemical Industries, and their stock solutions were made with a McIlvaine buffer (0.1 M citric acid and 0.2 M Na_2HPO_4, pH 5). All chemicals used in this study were of analytical reagent grade, and all solutions were prepared with milli-Q water. An F-kit for D-glucose and D-fructose determination was purchased from Roche Diagnostics K. K. The spectrophotometric measurement was performed using a Shimadzu multispec-1500 spectrophotometer. All measurements were performed in McIlvaine buffer (pH 5), and the temperature was controlled at 25 ± 2 °C using a thermostat.

Preparation of Carbon Particle-Modified Electrodes. Carbon particles used in this study were Ketjen Black (KB, EC 300J, Lion Corporation), Vulcan (XC-72R, Cabot Corporation), Carbon Nanoshere (CNS, Tokai Carbon Co., Ltd.), and Lamp Black 101 (LB, Tetsutani Co., Ltd.). Poly(vinylidene difluoride) (PVDF, molecular weight of 534000, Sigma-Aldrich Co.) was used as a binder and dissolved in *N*-methyl-2-pyrrolidone (Wako Pure Chemical Industries, Ltd.) as a 10% (w/w) solution. The carbon particles were ground with an agate triturator and then mixed with the PVDF solution to prepare carbon particle slurry. The weight ratio of PVDF to carbon particles was adjusted to be 2:8. The slurry was applied to the surface of carbon paper (CP, TGP-H-120, Toray Industries, Inc.) and dried in a drying oven at 60 °C for over 12 h. In coulometric measurements, KB-modified CP electrode (CPE) was dipped into the McIlvaine buffer containing FDH (ca. 30 μM) overnight at 4 °C and used as a working electrode.

Electrochemical Measurements. Cyclic voltammetry was performed using a BAS CV-50W electrochemical analyzer (BAS Inc.), using carbon particle-modified CPEs (6 mm diameter) without FDH as working electrodes. A platinum wire and Ag|AgCl electrode were used as counter and reference electrodes, respectively. The electrolysis solution was the McIlvaine buffer in a total volume of 2 mL. After 200 mM D-fructose and 1.5 μM FDH were added into the solution, the catalytic current attributable to the oxidation of D-fructose was observed.

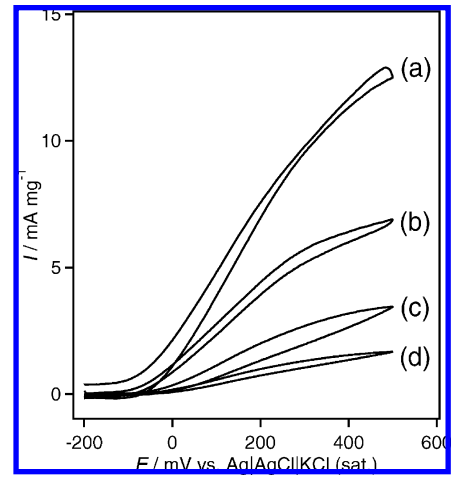

Figure 1. Cyclic voltammograms of FDH-catalyzed D-fructose oxidation at several carbon particle-modified electrodes ((a) KB, (b) Vulcan, (c) CNS, (d) LB) in pH 5 buffer in the presence of 200 mM D-fructose at room temperature. The voltammograms were obtained at a scan rate of 20 mV s^{-1}.

Coulometry was also performed using a BAS CV-50W electrochemical analyzer. In order to achieve rapid electrolysis, we prepared a batch-type electrolysis cell (5 mm diameter) filled with KB-modified CPEs with FDH as working electrodes. A platinum mesh was connected to the working electrodes as a lead. A platinum wire and Ag|AgCl electrode were used as counter and reference electrodes, respectively, which were separated from the working electrode with a KCl salt bridge tube. Because FDH is completely insensitive to O_2, the cell was open to the air without any deaeration process or sealing. All measurements were carried out at an applied potential of +500 mV, and 1 μL of D-fructose solution was injected into the electrolyte solution. The total volume of the analytical solution was 10−20 μL.

RESULTS AND DISCUSSION

Comparison of Carbon Particles. Figure 1 shows cyclic voltammograms recorded using various carbon particle-modified electrodes in the presence of 200 mM D-fructose at room temperature. The *y*-axis expresses the catalytic oxidation current densities per weight of carbon particle. No catalytic current was observed in the absence of FDH with any carbon-modified electrode. After the addition of FDH into the buffer solution containing D-fructose, sigmoidal catalytic voltammograms were obtained, indicating that FDH works as an electrocatalyst for the oxidation of D-fructose without any electron transfer mediator. The onset of the FDH-catalyzing wave at carbon-modified CP electrodes was around the formal potential of the heme c site of FDH, 39 mV.[13] The oxidation current density increased very slowly with time under stirring until it reached its maximum value. This indicates that the adsorption process is very slow; consequently, KB-modified CPE for the following coulometric measurement was dipped in FDH solution overnight to archive sufficient FDH loading. The catalytic oxidation current density depended on the carbon particles: the highest current density was produced at the KB-modified electrode. The high current density per weight of KB may be due to its unique hollow structure, small primary

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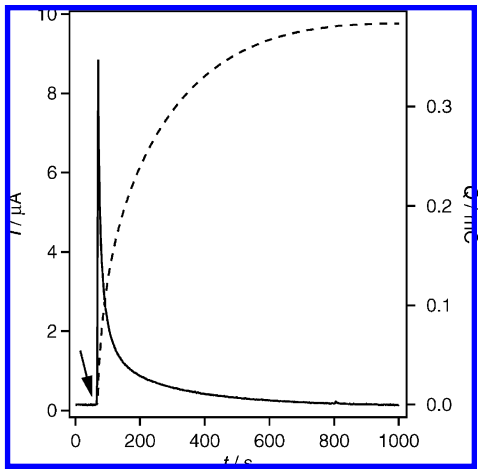

Figure 2. Time-dependent response curves of the current (solid line, left axis) and the electric charge (broken line, right axis). A 1-μL aliquot of 2 mM D-fructose was added at the point indicated by the arrow.

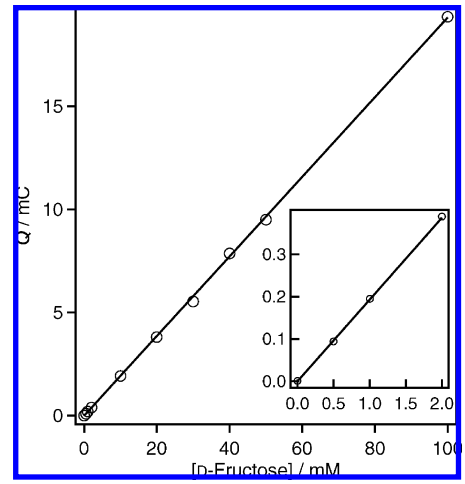

Figure 3. Coulometric response at +500 mV against the D-fructose concentration. One microliter each of D-fructose solution with the given concentration was electrolyzed. The open circles and the solid line show experimental value and theoretical value, respectively.

particle size (40 nm), and large specific surface area (around 800 $m^2\ g^{-1}$) compared to typical conductive carbon black. CNS and LB have large primary particle size (over 100 nm) and small specific surface area (around 20 $m^2\ g^{-1}$). The catalytic current density at the Vulcan-modified electrode was one-half of that at the KB-modified electrode. Vulcan also has small primary particle size (40 nm), but the specific surface area is not so large (200 $m^2\ g^{-1}$). The large specific surface for FDH adsorption and suitable morphology for electron transfer from FDH to electrode seem to be important factors for the fabrication of a DET-type biosensor electrode. The DET-based bioelectrocatalytic reaction would be correlated to the structure of the pore where the enzyme can be adsorbed. It has been reported that enzymes can be stabilized when they are immobilized in porous materials with matching between the pore size and the molecular size of the enzymes.[15−17] There is a possibility that not only the reactivity between enzymes and electrodes but also the stability of adsorbed enzymes would be improved if nanoporous carbons with suitable pore size are used. Therefore, in this study, KB was selected as a carbon particle for the following coulometric experiments.

Biocoulometry of D-Fructose. The solid and broken lines in Figure 2 show time dependence of the current and electric charge during the direct bioelectrocatalytic oxidation of D-fructose. When 1 μL of 2 mM D-fructose was added to the buffer solution, a rapid increase in the current was observed. After that, the current decreased back to the baseline as the reaction came to a completion. During the electrochemical measurement, the working electrode was polarized at +500 mV against the Ag|AgCl reference electrode to enhance the electrolysis rate according to the cyclic voltammograms in Figure 1. The electric charge was obtained by integrating the Faradaic current with respect to time from the injection to the end of the reaction. The electrolysis efficiency was obtained by dividing the electric quantity gained with coulometry by the theoretical value, which is calculated as *nFN*, according to Faraday's law, where *n*, *F*, and *N* are the number of electrons involved in the redox process (in case of D-fructose oxidation, $n = 2$), the Faraday constant, and the amount of D-fructose, respectively. The electrolysis efficiency was 99 ± 3% (n = 5, n: number of repetition). The values indicate successful determination of D-fructose with the coulometric biosensor. To the best of our knowledge, this is the first report of a coulometric biosensor based on DET. However, the electrolysis time is rather long. In order to shorten the electrolysis time, more effort will be needed to load increased concentration of enzyme with high electroactivity onto the electrode. The electrolysis time would also be shortened by increasing the A/V ratio, where A and V are the active surface area of the working electrodes and the cell volume (electrolyte solution), respectively. Chip type thin-layer electrolysis cell with suitable space between the carbon nano particles would allow the rapid electrolysis.

D-Fructose Concentration Dependence and the Sensor Stability. The electric charge obtained with the coulometric measurement is plotted against the D-fructose concentration in Figure 3. The electric charge in the coulometric biosensor showed a linear relationship against the D-fructose concentration over the range from 1 to 100 mM with a 1 μL injection. The open circles and the solid line show experimental and theoretical values, respectively. The electric charge gained by the coulometric measurement was in good agreement with the theoretical value at D-fructose concentration from 1 to 100 mM.

This result shows that the method is able to accurately measure the D-fructose concentration in the range of 1 to 100 mM. Further extension of this detection range will require a reduction of the noise (the lowest detection limit) and a shortening of the electrolysis time (the highest detection limit). It is noted that amperometric D-fructose biosensors reported previously have a linear response range to the D-fructose concentration below 10−20 mM[11,18−20] because of the relatively low K_M values for D-fructose (10 mM).[7,8]

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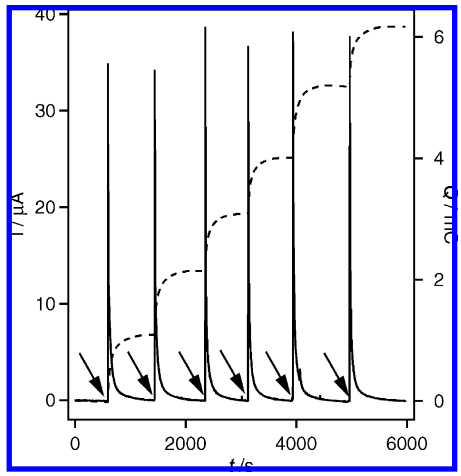

Figure 4. Time-dependent response curves of the current (solid line, left axis) and the electric charge (broken line, right axis). Five nanomoles each of D-fructose (0.5 μL, 10 mM) was successively syringed at the points indicated by the arrows.

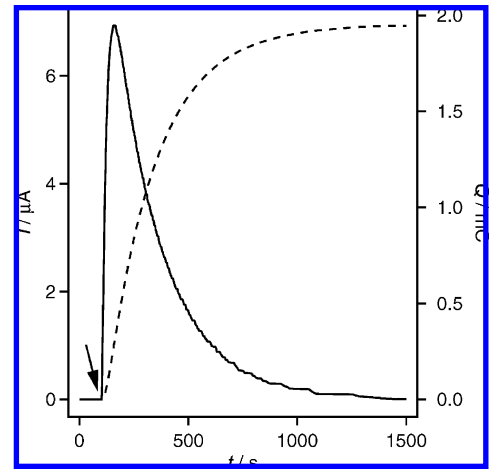

Figure 5. Time-dependent response curves of the current (solid line, left axis) and the electric charge (broken line, right axis). A 1-μL aliquot of 10 mM sucrose was added at the point indicated by the arrow.

Although stability and reusability are not essential for current electroenzymatic sensors, being inexpensive enough to be disposable, they may be required for a future generation biosensor. We tested the stability of our FDH-adsorbed KB-modified CPEs by checking the coulometric response for D-fructose everyday for 5 days, and the electrodes were stored in a refrigerator at 4 °C when not in use. The coulometric biosensor responses were maintained in all subsequent experiments. The sensor also showed a good stability during successive operation. Figure 4 shows the time-dependent response curves of the current (solid curve, left axis) and the electric charge (broken curve, right axis) obtained with controlled-potential electrolysis of 5 nmol each of D-fructose (0.5 μL, 10 mM) successively syringed at the points indicated by the arrows. Coulometric response at each injection was maintained constant. Good stability is most likely due to a carbon with suitable porous structure and surface for DET-type bioelectrocatalysis of FDH, minimizing the enzyme denaturation and desorption. Furthermore, coulometric technology, which is not inherently affected by the kinetics of the enzymatic reaction, would be responsible for the stable responses.

Biocoulometry of Sucrose. DET-based biocoulometry may become applicable to broader areas if it proves reliable in determining not only D-fructose but also oligo/polysaccharides containing D-fructose units. In this work, sucrose was tested as an example and its concentration was measured based on the coulometric measurement of D-fructose generated through hydrolysis by a specific hydrolase, invertase. Coulometric determination of sucrose was done by using the KB-modified CPE in combination with FDH and invertase. An excess amount of invertase was added to the KB-modified electrode with FDH in order to increase the sucrose hydrolysis rate. When 1 μL of a sample solution containing 10 mM sucrose was injected into the electrolysis cell, hydrolyzed D-fructose was electrolyzed at an electrolysis efficiency of 100 ± 5% (n = 5) (Figure 5). This result indicates that sucrose was completely hydrolyzed to D-fructose and glucose with the aid of invertase. A high surface area of the porous carbon structure would be capable of immobilizing large amounts of enzymes with ease. It can be expected that not only sucrose but also other oligo/polysaccharides containing D-fructose units would be detected after suitable hydrolysis. Another example would be a clearance test of the kidney[21] using inulin,[22] which is a fructo-polysaccharide, and can be determined as the D-fructose concentration using a specific hydrolase, inulinase.[23]

Removal of Electroactive Interfering Compound. There are many electroactive compounds such as ascorbate in real samples which are directly oxidized at the electrodes and prevent accurate measurements. It is possible to minimize the effect of electrolysis of interfering electroactive chemicals by operating at a low electrode potential (around 0 V). In MET-type bioelectrocatalysis, the electrolysis potential can be controlled by selecting a mediator with lower formal potential.[24] In the case of DET-type biocoulometry catalyzed by FDH, it will take much longer time to complete the electroenzymatic oxidation of D-fructose at 0 V because of the low catalytic current density, as shown in Figure 1. Another approach to overcome the problem of the electrochemical oxidation of interfering compounds is to employ polymeric membranes to eliminate them by size exclusion or electrostatic repulsion. Although such membranes are sometimes useful for amperometric biosensors, there are critical problems for their use for coulometric biosensors such as the immobilization of the membranes onto the electrodes and, more importantly, the inhibition of the mass transfer.

The proposed method described in this paper is based on independently measuring the electric charge generated from known interfering compounds by using a KB-modified CPE without FDH and subsequently subtracting it from the total charge obtained at the FDH-modified electrode (Figure 6). In this study, ascorbate was taken as a typical interfering compound. The test solution consisted of 1 mM ascorbate and 10 mM D-fructose in McIlvaine buffer (pH 5). The solid line and the broken line show the time dependence of the total electric charge and the charge of ascorbate alone, respectively. The electrolysis efficiency of D-fructose was 99 ± 4% (n = 5). The effect of electrolysis of

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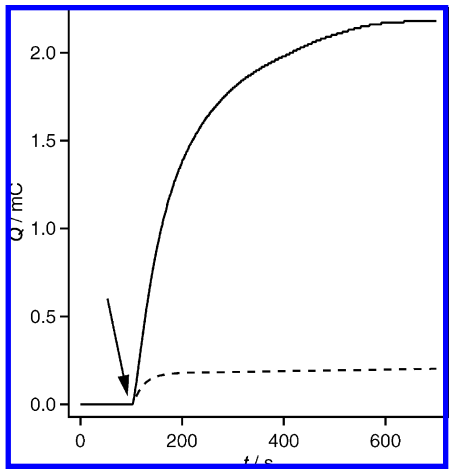

Figure 6. Time-dependent response curves of the total electric charge (solid line, left axis) and the electric charge of ascorbate (broken line, right axis). A one microliter aliquot containing 1 mM ascorbate and 10 mM D-fructose was syringed into the electrolysis cell.

Table 1. Comparison of D-Fructose Concentration in Beverage Samples Determined by the Coulometric Sensor and the Enzyme Kit

sample	coulometry (mM)	F-kit method (mM)
apple juice	336 ± 9 (n = 5)	322 ± 13 (n = 3)
cola	267 ± 6 (n = 3)	270 ± 0.3 (n = 2)
sports drink	180 ± 3 (n = 6)	188 ± 9 (n = 4)

ascorbate can be successfully removed with this correction method based on coulometric charges (not current value). This method can easily be extended to other electroactive compounds.

Biocoulometry of D-Fructose in Beverages. The fructose concentration in several beverages was determined with the proposed coulometric method as well as and F-kit as a reference method. The F-kit method is based on the spectrophotometrical detection of NADPH formed in a multiple enzymatic reaction at 340 nm. This method is practically insensitive to electroactive compounds. Table 1 shows the concentration of D-fructose in the commercial beverages determined by both of the coulometric and the F-kit methods. The results of the DET-based enzymatic coulometry agreed well with the values obtained with the F-kit method. As was mentioned in the introduction, FDH exhibits extremely high substrate specificity for D-fructose and are not inhibited by substrate analogues such as D-glucose, D-mannose, and D-fructose phosphate, and therefore it is possible to determine the D-fructose concentration in the presence of various substances at high concentrations. The F-kit measurement requires three pretreatments steps to complete all the reactions and blank measurements. It is somewhat time-consuming and expensive. The operation of this coulometric measurement is much simpler and easier than the photometric method. Additionally, the concentration range to be measured of this method is much wider than that of F-kit (from about 0.2 to 2.7 mM).

CONCLUSION

A coulometric D-fructose biosensor based on DET-based reaction has been developed using a KB-modified CPE as a working electrode. The current density of D-fructose oxidation depended on the surface structure of carbon electrode and increased significantly by modifying KB on the electrode. This method was able to successfully detect D-fructose at a wide concentration range from 1 to 100 mM, which is much wider than that guaranteed by the amperometric methods reported to date. The combination of oxidoreductase and hydrolase could open the way to the determination of a wide variety of oligo/polysaccharides. In addition, the interference of electroactive compounds such as ascorbate was successfully removed with a simple detection procedure. Determination of D-fructose in beverage samples was also possible using this coulometric biosensor without interference from electroactive compounds. Miniaturization of the test solution volume using microscale chips, such as the blood glucose sensor chip, would be the next step toward practical use as a field device.

ACKNOWLEDGMENT

The authors thank Lion Corporation and Prof. Abe in Kyoto University for their kind gifts of KB and CNS, respectively. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan [19310070 (to K. Kano)].

Received for review August 6, 2009. Accepted October 3, 2009.

AC901771T