JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Protective Effects of Quercetin and Vitamin C against Oxidative Stress-Induced Neurodegeneration

HO JIN HEO AND CHANG YONG LEE*

Department of Food Science and Technology, Cornell University, Geneva, New York 14456

Clinical trials of several neurodegenerative diseases have increasingly targeted the evaluation of various antioxidants' effectiveness. The human diet contains several thousand phytochemicals, many of which have significant bioactivities. Vitamin C, a naturally occurring antioxidant, is known to reduce the risk of neurodegenerative disorders such as Alzheimer's disease. Quercetin, one of the major flavonoids in some fruits and vegetables, has much stronger antioxidative and anticarcinogenic activities than vitamin C. Therefore, we investigated the protective effects of quercetin on hydroxy peroxide-induced neurodegeneration. To determine the protective effects, PC12 cells were preincubated with quercetin and vitamin C before H_2O_2 treatment for 2 h. Results showed that cell viability was clearly improved with quercetin, and quercetin showed a higher protective effect than vitamin C. Because oxidative stress is known to increase neuronal cell membrane breakdown, we further investigated lactate dehydrogenase and trypan blue exclusion assays. We observed that quercetin decreased oxidative stress-induced neuronal cell membrane damage more than vitamin C. These results suggest that quercetin, in addition to many other biological benefits, contributes significantly to the protective effects of neuronal cells from oxidative stress-induced neurotoxicity, such as Alzheimer disease.

KEYWORDS: Neurodegeneration; oxidative stress; quercetin; vitamin C

INTRODUCTION

The deleterious influence of oxidative stress has long been known to induce neurodegenerative disorders. There is evidence that oxidative stress including free radicals plays a key role in Alzheimer's disease (AD) and Parkinson's disease (PD) (*1*−*3*). The physiological function of oxidative stress for neurodegenerative diseases is that cumulative oxidative damage could be responsible for the late life onset and the slowly progressive nature of these chronic disorders. The central nervous system (CNS) in the human brain is especially vulnerable to free radical attack. The main energy supply of this highly oxygenated organ is the oxidative metabolism of the mitochondrial respiratory action. In oxidative stress, the superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) produced by living tissue cannot be readily neutralized because of low catalase, superoxide dismutase (SOD), and glutathion peroxidase activities present in the brain. Moreover, brain membrane lipids are very rich in polyunsaturated fatty acids, which are especially sensitive to free radical-induced lipid peroxidation (*1*). Among reactive oxygen species (ROS), $O_2^{\bullet-}$ and H_2O_2 are the two major oxidative stress inducers. Although H_2O_2 is a reactive nonradical molecule, it can easily permeate through biological cell membranes, while $O_2^{\bullet-}$ can only move through an anion channel (*2*).

Recently, natural food-derived components such as vitamins and phytochemicals have received great attention because they are perceived as safe, functional foods. Vitamin C is a water-soluble antioxidant. Several studies showed that a high dietary intake of vitamin C decreased the subsequent risk of AD (*4*) and a higher plasma concentration of vitamin C was associated with better memory performance (*5*). Flavonoids are a large heterogenic group of benzo-γ-pyron derivatives, which are abundantly present in food products and beverages derived from fruits and vegetables. It has been proposed that phenolic phytochemicals exert positive health effects in chronic disease states, including cancer and neurodegenerative disorders. Many physiological benefits of flavonoids have been attributed to their antioxidant and free radical scavenging properties (*6*). Flavonol quercetin is one of the most frequently researched flavonoids. Quercetin has been thoroughly investigated for its abilities to express antiproliferative effects (*7*). In our previous studies, we reported that apple phenolics have strong antioxidative and antiproliferative activities due to their synergistic effects and that vitamin C has strong antioxidative and anticarcinogenic activities (*8*−*10*). However, there are relatively limited research papers on quercetin and vitamin C that relate to neurodegenerative disorders such as AD. In the present study, possible protective effects of quercetin and vitamin C on H_2O_2-induced neuronal cells cytotoxicity were investigated.

* Author to whom correspondence should be addressed [telephone +1-315-787-2271; fax +1-315-787-2284; e-mail cyl1@cornell.edu].

10.1021/jf049243r CCC: $27.50
Published on Web 11/10/2004

MATERIALS AND METHODS

Materials. RPMI 1640 medium, fetal bovine serum, horse serum, penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY). Vitamin C was purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals were the products of Sigma (St. Louis, MO). Stock solutions of quercetin (10−100 μmol/L) and vitamin C (10−100 μmol/L) were prepared in dimethyl sulfoxide (DMSO) and deionized distilled water, respectively. Final DMSO concentration never exceeded 0.5% (v:v) in all treatment groups. Controls were not pretreated with quercetin or vitamin C, and cells were preincubated with quercetin and vitamin C for 10 min and then treated with H_2O_2 (400 μmol/L) for 2 h.

Cell Culture. PC12 cells were propagated in RPMI 1640 medium containing 10% heat-inactivated horse serum, 5% fetal bovine serum, and 50 units/mL penicillin and 100 μg/mL streptomycin in a humidified incubator at 5% CO_2 (*11*). The PC12 cell line was derived from transplantable rat pheochromocytoma. The cells respond reversibly to nerve growth factor by induction of the neuronal phenotype.

Determination of Cell Viability. PC12 cells were plated at a density of 10^4 cells/well on 96-well plates in 100 μL of RPMI, and the cell viability was determined by the conventional MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction assay (*12*). Cells were incubated with 0.25 mg of MTT/mL (final concentration) for 2 h at 37 °C, and the reaction was stopped by adding a solution containing 50% dimethylformide and 20% sodium dodecyl sulfate (pH 4.8). The amount of MTT formazan product was determined by measuring absorbance using a micro-plate reader (Dynex Technologies, Chantilly, VA) at a test wavelength of 570 nm and reference wavelength of 630 nm.

Measurement of Cell Membrane Toxicity. Lactate dehydrogenase (LDH) assay measures either the number of cells via total cytoplasmic LDH or the membrane integrity. PC12 cells were pretreated with quercetin, and H_2O_2 was added at a final concentration of 400 M for 2 h. Cells were precipitated by centrifugation at 2000*g* for 2 min at room temperature, 50 μL of the supernatants was transferred into new wells, and LDH was determined using the in vitro toxicology assay kit (Sigma, St. Louis, MO). Plasma membrane damage was evaluated by measuring the amount of the intracellular enzyme LDH released into the medium.

The trypan blue exclusion assay was based on the capability of viable cells to exclude the dye. Because viable PC12 cells maintained membrane integrity, the cells did not allow trypan blue dye to pass through the cell membrane. Cells with damaged membrane appeared blue due to dye accumulation and were counted as dead. The dye, 0.4% trypan blue, was added to PC12 cells, and after 5 min cells were loaded into a hematocytometer and counted for dye uptake. The number of viable cells was calculated as a percent of the total cell population (*12*).

Statistical Analysis. Data were analyzed using the Sigma Plot software. All data were expressed as mean ± SD. Statistical comparisons within the same group were performed for paired observations. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Neurodegenerative disorders including AD and PD represent a major burden on our society, whether it is from normal aging or as a consequence of a neurodegenerative disorder. These chronic diseases resulting in memory loss are often associated with a loss of independent functioning. Furthermore, the possibility that nutritional deficiencies may play a role in cognitive deficits has been considered for several years. Therefore, the development of potentially useful protective factors for memory deficits or dementia is becoming an increasingly important subject (*13*).

Because direct addition of the peroxidizing agent, H_2O_2, induces neuronal cell death, the protective efficacy of quercetin and vitamin C was evaluated by exposing PC12 cells to H_2O_2 using MTT reduction assay. This test is used as a marker for cell viability, because intact mitochondrial enzymes can only reduce MTT (**Figure 1**). Hydrogen peroxide (400 μM) caused

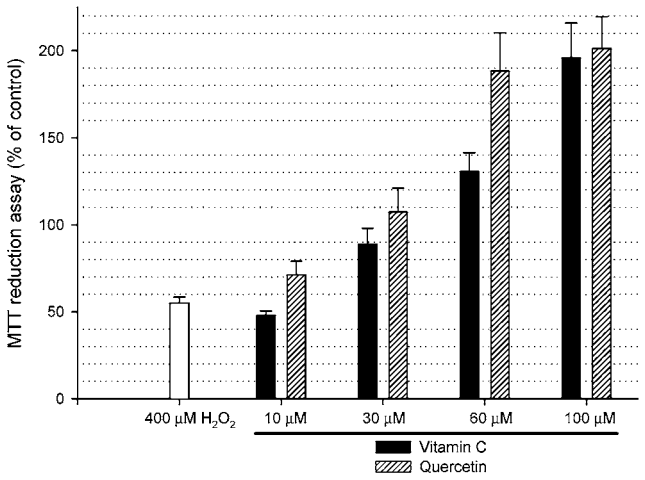

Figure 1. Cell protective effect of quercetin and vitamin C on H_2O_2-induced cytotoxicity in the PC12 cell system. PC12 cells were pretreated for 10 min with various concentrations. After 10 min, the cells were treated with 400 μM H_2O_2 for 2 h. Levels of cell viability were measured using the MTT assay as described in Materials and Methods. The viability of untreated control cells was defined as 100%. Results shown are means ± SD ($n = 3$). A significant difference ($P < 0.05$) was observed on the H_2O_2-induced neurotoxicity.

a significant decrease in cell viability (44 ± 6.6%), but preincubation with quercetin and vitamin C protected H_2O_2-induced toxicity in PC12 cells in a dose-dependent manner. Neuronal cell protective effects were observed over 10 μM quercetin, and it showed a higher cell viability effect over 30 μM (**Figure 1**). In contrast, the protective effects of vitamin C were lower than those of quercetin (10−60 μM), but both showed almost the same protective effects at the highest concentration (100 μM). These data agree with our previous studies that quercetin has a higher antioxidative activity than vitamin C (*8*).

One of the most sensitive primary targets of oxidative stress may be mitochondria in neuronal cells (*14*). Mitochondrial DNA does not encode for any repair enzymes, and, unlike nuclear DNA, it is not shielded by protective histones. It has been suggested that there are mitochondrial defects in the pathogenesis of an AD patient (*15*). In the present study, we have demonstrated that H_2O_2-induced apoptosis in PC12 cells was suppressed by pretreatment with quercetin and vitamin C. Hydrogen peroxide caused a decrease in MTT reduction in PC12 cells, which was partly restored in the presence of quercetin and vitamin C. These data suggest that PC12 cell protection by quercetin and vitamin C is partially due to mitochondrial protection mechanisms.

It has been reported that superoxide dismutase and hemoxygenase-1, biomarkers of oxidative stress, were promoted by amyloid β (Aβ)/H_2O_2-induced oxidative stress in aged transgenic mice and PC12 cells (*16*). This Aβ protein decreases MTT dye reduction by an increased instability of the plasma membrane (*17*) and has been identified as a possible source of oxidative stress in the AD brain because it can acquire a free radical state contributing to its toxic effects (*16*). In this respect, we have assessed the protective effect of quercetin and vitamin C on H_2O_2-induced membrane toxicity using the LDH release assay. Preincubation with quercetin showed more efficient inhibitory activity than vitamin C at all concentrations (**Figure 2A**). To confirm whether quercetin and vitamin C block the H_2O_2-induced membrane damage, the trypan blue exclusion assay was also used. This assay directly measures viable cells maintaining

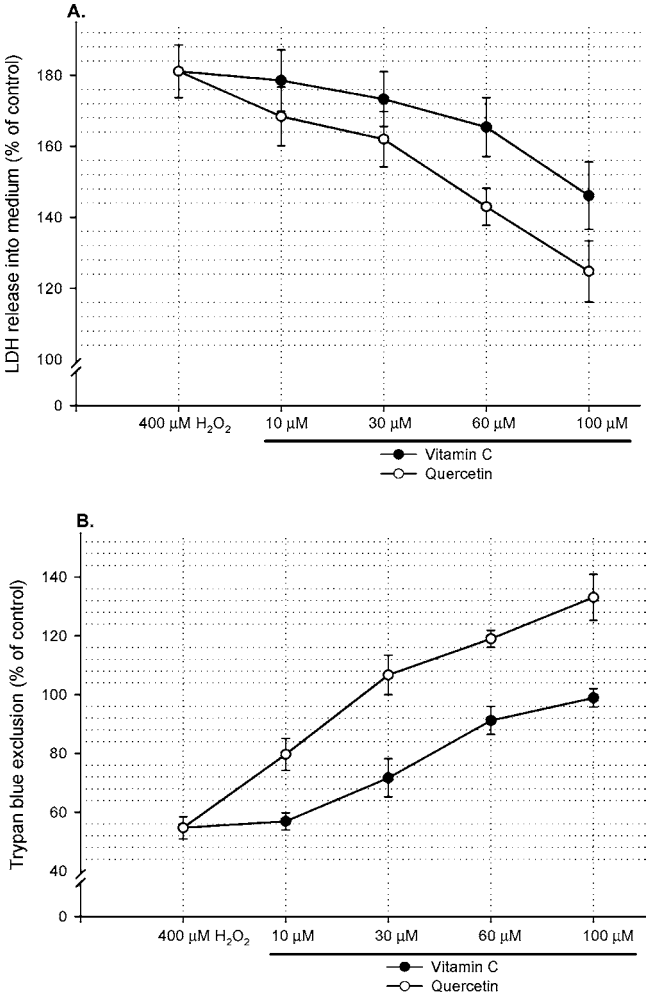

Figure 2. Effects of quercetin and vitamin C on H_2O_2-induced membrane damage in PC12 cells. PC12 cells were pretreated for 10 min with various concentrations. The cells were treated with H_2O_2 (400 μM) for 2 h. (**A**) LDH activity in culture supernatants was measured with a colorimetric LDH assay kit. (**B**) Inhibition of H_2O_2-induced cytotoxicity in PC12 cells after pretreatment with quercetin and vitamin C as assessed by trypan blue exclusion staining followed by cell counting. The viability of untreated control cells was defined as 100%. All data are represented as the means ± SD ($n = 3$) and values obtained from three separate cultures. Statistical analysis indicated that the influence of the compounds used had a significant effect on H_2O_2-induced membrane toxicity (LDH release and trypan blue exclusion) ($P < 0.05$).

the capability of excluding the dye and may reflect more precisely the stability of viable cell membrane. H_2O_2-induced oxidative stress increased plasma membrane damage, but quercetin and vitamin C protected the PC12 cells in a dose-dependent manner (**Figure 2B**). Our data also showed that quercetin has more cell protective effect than vitamin C. These results suggest that quercetin and vitamin C with excellent antioxidant activities could protect the neuronal cell membrane against H_2O_2-induced neurotoxicity, although the protective effect of vitamin C was not strong as compared to that of quercetin.

Lipid peroxidation is increased in neurodegenerative diseases such as AD. Polyunsaturated fatty acid levels in neuronal cell membrane, especially arachidonic acid and docosahexaenoic acid that are more vulnerable to attack by ROS, are decreased. Lipid peroxidation can lead to changes in membrane integrity and fluidity (*18*). The 3-OH in the flavonoids' AC-ring is important for antioxidant activity, which can be boosted by

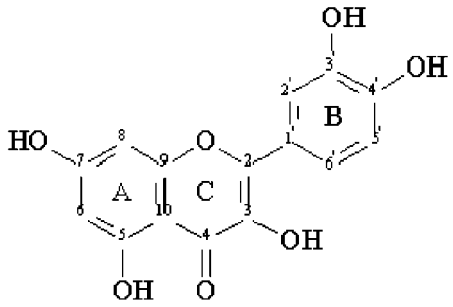

Figure 3. Structure of quercetin ($C_{15}H_{10}O_7$).

electron-donating substituents (e.g., OH- or OCH_3-group) at the 5 and 7 positions. Moreover, flavonoids with a catechol structure in ring B are very beneficial inhibitors of lipid peroxidation, and this moiety is very important to the efficacy of cell protection (**Figure 3**) (*19*). Consistent with this notion, quercetin was found to prevent lipid peroxidation via generation of oxidative stress (*19*). Our data suggest that the strong protective effect of quercetin on oxidative stress-induced membrane damage may be due to the inhibition of lipid peroxidation.

In a previous study, quercetin protects the mouse hippocampal cell line HT-22 from glutamate-induced oxidative toxicity by two distinct mechanisms preventing intracellular glutathione (GSH) and blocking ROS production (*20*). The loss in GSH up to 85% of the control level causes only a 5−10-fold increase in levels of ROS (*21*). A greater GSH loss stimulates mitochondria to produce a 100-fold increase in ROS, resulting in programmed cell death (*21*). Quercetin increases levels of GSH in HT-22 cells in the presence of glutamate (*20*). The structural determinants required to protect neuronal cell from oxidative stress are as follows: the presence of the hydroxyl group on the C3 position, an unsaturated C ring, and hydrophobicity (**Figure 3**) (*22*). The hydroxyl group on the C-3 position dramatically alters the protective effect on cell viability. The unsaturation of the C ring in flavonoids is essential for protection from oxidative glutamate toxicity and allows the electron delocalization across the molecule for the stabilization of the free radical, and is also an important factor for antioxidants in the cell-free systems. The decrease in hydrophobicity by glycosylation (rutin) or ionization (cyanidin) inactivates the protective activities on oxidative glutamate toxicity (*22*). In addition, a hydrophobic antioxidant may easily pass into the cytoplasm where ROS are generated and accumulate in oxidative glutamate toxicity (*20*). Quercetin has the specific structure and the preventing effect on GSH reduction to protect oxidative stress-induced neurotoxicity as previously mentioned. Our data showed that the protective effect of quercetin was more efficacious than that of vitamin C. This may be due to the structural property and variable physiological benefits of quercetin. In addition, the blood−brain barrier (BBB), formed by the endothelium of brain microvessels, is a regulatory interface and selectively limits drug delivery to the CNS. Hence, drug permeability into brain will be controlled by its physicochemical characteristics such as hydrophobicity or lipophilicity (*23*). It has been reported that quercetin can flux into brain regions (*24*). Therefore, it is possible that quercetin with beneficial antioxidant and biological functions is able to penetrate the BBB and can protect the H_2O_2-induced cytotoxicity.

ABBREVIATIONS USED

AD, Alzheimer's disease; BBB, blood−brain barrier; CNS, central nervous system; GSH, intracellular glutathione; LDH,

lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; SOD, superoxide dismutase

LITERATURE CITED

(1) Floyd, R. A. Antioxidants, oxidative stress, and degenerative neurological disorders. *Exp. Biol. Med*. **1999**, *222*, 236−245.

(2) Multhaup, G.; Ruppert, T.; Schlicksupp, A.; Hesse, L.; Beher, D.; Masters, C. L.; Beyreuther, K. Reactive oxygen species and Alzheimer's disease. *Biochem. Pharmacol*. **1997**, *54*, 533−539.

(3) LeWitt, P. A. New drugs for the treatment of Parkinson's disease. *Pharmacotheraphy* **2000**, *20*, 26S−32S.

(4) Masaki, K. H.; Losonczy, K. G.; Izmirlian, G.; Foley, D. J.; Ross, G. W.; Petrovitch, H.; Havlik, R.; White, R.; White, L. R. Association of vitamin E and C supplement use with cognitive function and dementia in elderly men. *Neurology* **2000**, *54*, 1265−1272.

(5) Perrig, W. J.; Perrig, P.; Stahelin, H. B. The relation between antioxidants and memory performance in the old and very old. *J. Am. Geriatr. Soc*. **1997**, *45*, 718−724.

(6) Rice-Evans, C. Flavonoid antioxidants. *Curr. Med. Chem*. **2001**, *8*, 797−807.

(7) Csokay, B.; Prajda, N.; Weber, G.; Olah, E. Molecular mechanism in the antiproliferative action of quercetin. *Life Sci*. **1997**, *60*, 2157−2163.

(8) Eberhardt, M. V.; Lee, C. Y.; Liu, R. H. Antioxidant activity of fresh apples. *Nature* **2000**, *405*, 903−904.

(9) Lee, K. W.; Lee, H. J.; Kang, K. S.; Lee, C. Y. Preventive effects of vitamin C on carcinogenesis. *Lancet* **200**2, *359*, 172.

(10) Kim, D. O.; Lee, K. W.; Lee, H. J.; Lee, C. Y. Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals. *J. Agric. Food Chem*. **2002**, *50*, 3713−3717.

(11) Heo, H. J.; Cho, H. Y.; Hong, B. S.; Kim, H. K.; Heo, T. R.; Kim, E. K.; Kim, S. K.; Kim, C. J.; Shin, D. H. Usolic acid of *Origanum majorana* L. reduces Aβ-induced oxidative injury. *Mol. Cells* **2002**, *13*, 5−11.

(12) Heo, H. J.; Cho, H. Y.; Hong, B. S.; Kim, H. K.; Kim, E. K.; Kim, B. K.; Shin, D. H. Protective effect of 4′,5-dihydroxy-3′,6,7-trimethoxyflavone from *Artemisia asiatica* against Aβ-induced oxidative stress in PC12 cells. *Amyloid* **2001**, *8*, 194−201.

(13) Youdim, K. A.; Joseph, J. A. Possible emerging role of phytochemicals in improving age related neurological dysfunctions: a multiplicity of effects. *Free Radical Biol. Med*. **2001**, *30*, 583−594.

(14) Carmichael, J.; De Graff, W. G.; Gadzar, A. F.; Minna, J. D.; Mitchell, J. B. Evaluation of tetrazolium-based semi-automated colorimetric assay: assessment of chemosensitive testing. *Cancer Res*. **1987**, *46*, 936−941.

(15) Wallace, D. C. Mitochondrial genetics: a paradigm for aging and degenerative disease? *Science* **1992**, *256*, 628−632.

(16) Shoffner, J. M.; Brown, M. D.; Torroni, A.; Lott, M. T.; Cabell, M. F.; Mirra, S. S.; Beal, M. F.; Yang, C. C.; Gearing, M.; Salvo, R. Mitochondrial DNA variants observed in Alzheimer's disease and Parkinson disease patients. *Genomics* **1993**, *17*, 171−184.

(17) Pappolla, M. A.; Chyan, Y. J.; Omar, R. A.; Hsiao, K.; Perry, G.; Smith, M. A.; Bozner, P. Evidence of oxidative stress and *in vivo* neurotoxicity of beta-amyloid in a transgenic mouse model of Alzheimer's disease: a chronic oxidative paradigm for testing anti-oxidant therapies in vivo. *Am. J. Pathol*. **1998**, *152*, 871−877.

(18) Prasad M. R.; Lvell, M. A.; Yatin, M.; Dhillon, H. S.; Markesbery, W. R. Regional membrane phospholipid alteration in Alzheimer's disease. *Neurochem. Res*. **1998**, *23*, 81−88.

(19) Heijnen, C. G.; Haenen, G. R.; Oostveen, R. M.; Stalpers, E. M.; Bast, A. Protection of flavonoids against lipid peroxidation: the structure activity relationship revisited. *Free Radical Res*. **2002**, *36*, 575−581.

(20) Ishige, K.; Schubert, D.; Sagara, Y. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. *Free Radical Biol. Med*. **2001**, *30*, 433−446.

(21) Tan, S.; Sagara, Y.; Liu, Y.; Maher, P.; Schubert, D. Peroxide production in oxidative glutamate toxicity. *J. Cell Biol*. **1998**, *141*, 1423−1432.

(22) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activityrelationships of flavonoids and phenolic acids. *Free Radical Biol. Med*. **1996**, *20*, 933−956.

(23) Yodium, K. A.; Dobbie, M. S.; Kuhnle, G.; Proteggente, A. R.; Abbott, N. J.; Rice-Evans, C. Interaction between flavonoids and the blood-brain barrier: *in vitro* studies. *J. Neurochem*. **2003**, *85*, 180−192.

(24) Yodium, K. A.; Zeeshan Qaiser, M.; Begley, D. J.; Rice-Evans, C. A.; Abbott, N. J. Flavonoid permeability across an in situ model of the blood-brain barrier. *Free Radical Biol. Med*. **2004**, *5*, 592−604.

Received for review May 11, 2004. Revised manuscript received September 8, 2004. Accepted September 14, 2004. This work was supported in part by the Postdoctoral Fellowship Program of the Korea Science and Engineering Foundation (2003) for H.J.H. at Cornell University.

JF049243R