Magnoflorine from *Coptidis Rhizoma* Protects High Density Lipoprotein during Oxidant Stress

Tran Manh HUNG,^a Jong Pill LEE,^d Byung Sun MIN,^b Jae Sue CHOI,^c MinKyun NA,^a XinFeng ZHANG,^a Tran Minh NGOC,^a IkSoo LEE,^a and KiHwan BAE^{*,a}

^aCollege of Pharmacy, Chungnam National University; Daejeon 305–764, Korea; ^bCollege of Pharmacy, Catholic University of Daegu; Gyeongsan 712–702, Korea; ^cFaculty of Food Science and Biotechnology, Pukyong National University; Busan 608–737, Korea; and ^dKorea Food and Drug Administration; Seoul 122–704, Korea.

Received January 8, 2007; accepted March 6, 2007

The objective of the present study was to investigate the beneficial properties of magnoflorine, an alkaloid isolated from *Coptidis Rhizoma*, on protecting human high density lipoprotein (HDL) against lipid peroxidation. Magnoflorine exerts an inhibitory effect against Cu²⁺-induced lipid peroxidation of HDL, as showed by prolongation of lag time from 62 to 123 min at the concentration of 3.0 μM. It also inhibits the generation of thiobarbituric acid reactive substances (TBARS) in the dose-dependent manners with IC₅₀ values of 2.3±0.2 μM and 6.2±0.5 μM since HDL oxidation mediated by either catalytic Cu²⁺ or thermo-labile radical initiator (AAPH), respectively. Separately, Cu²⁺ oxidized HDL lost the antioxidant action but the inclusion of magnoflorine/Cu²⁺ oxidized HDL can protect LDL oxidation according to increasing magnoflorine concentration. The results suggest that magnoflorine may have a role to play in preventing the HDL oxidation.

Key words *Coptidis Rhizoma*; magnoflorine; cardiovascular disease; high density lipoprotein; antioxidant

Blood levels of high-density lipoprotein (HDL) are inversely associated with risk for coronary heart disease,¹⁾ the most common cause of death. Among the potential mechanisms by which HDL may exert its cardio-protective effect, much attention has recently focused upon its antioxidant function which protects low-density lipoprotein (LDL) from oxidative (*i.e.*, atherogenic) modification.^{2,3)} Numerous prospective cohort studies have demonstrated the protective nature of an elevated level of HDL, and the high risk associated with low levels of this class of lipoprotein.^{4,5)} The decrease in atherosclerosis associated with increased HDL levels in human apolipoprotein A-I (apoA-I) transgenic animals further illustrates the protective nature of HDL.^{6–8)} HDL is susceptible to oxidation by a variety of pro-oxidants.⁹⁾ During incubation with redox-active copper ions (Cu²⁺), HDL is found to undergo extensive lipid oxidation as indicated by a loss in polyunsaturated fatty acyl content and concurrent accumulation of lipid epoxides, lyso-phosphatidylcholine, and cholesterol oxidation products; thiobarbituric acid-reactive substances, a marker of lipid oxidation, similarly increase in HDL incubated with Cu²⁺.^{10,11)} In general, oxidation of HDL is found to result in a loss of cardio-protective properties. For example, the antioxidant ability of HDL to protect LDL from atherogenic modification is lost upon Cu²⁺-mediated oxidation of HDL.^{12,13)}

As a part of our screening program to find antioxidant compounds from natural sources, we have attempted to determine the human density lipoproteins oxidation inhibitory constituents of medicinal plants in Korea. *Coptidis Rhizoma* (root of *Coptis chinensis* FRANCH, Ranunculaceae) has been used to treat diabetes mellitus for more than 1000 years in the history of Chinese medicinal remedy. It has long been used for treating gastroenteritis, diarrhea, anti-tumor, and antimicrobial.^{14–17)} *Coptidis Rhizoma* is currently used for several skin diseases including acne, neurodermatitis, skin ulcers,¹⁸⁾ and dermatological indications.¹⁹⁾ Moreover, *Coptidis Rhizoma* is prescribed as a crude drug in folk medicines,

such as Oren-gedoku-to and San'o-shashinto, for several vascular disorders. It was useful as an alternative therapy for hypercholesterolemia, effective in preventing hypercholesterolemic atherosclerosis and lowering the relative risk of coronary artery disease through decreases in lipid peroxidation and cholesterol levels.²⁰⁾ The alkaloids present in *Coptidis Rhizoma* (berberine, coptisine, jateorrhizine and palmatine...) were considered to be its active constituents.^{21,22)} Among them, magnoflorine is a quaternary alkaloid with the isoquinoline skeleton. Magnoflorine was showed to suppress the induction phase of the cellular immune response,²³⁾ and assigned as noncytotoxic to various human cancer cell lines.²⁴⁾ The antioxidant activity of magnoflorine was investigated with respect to its structural feature and physico-chemical properties to inhibit free radical, peroxidation of dioleoyl phosphatidylcholine, and liposomes initiated by thermal degradation of the azoinitiator (AAPH).²⁵⁾ However, no studies have been specifically investigated the ability of this antioxidant to protect human density lipoprotein from oxidation. In this study, we examined the susceptibility of HDL to *in vitro* Cu²⁺ and AAPH induced lipid peroxidation in the presence of magnoflorine. Also, the study of magnoflorine/Cu²⁺ oxidized HDL in preventing LDL oxidation was used to investigate whether the inclusion protects LDL from oxidative modification.

MATERIALS AND METHODS

Plant Material The root of *Coptis chinensis* FRANCH (*Coptidis Rhizoma*) was purchased from a local market in Daejeon, Korea in June 2006, and was identified by Prof. KiHwan Bae. A voucher specimen (CNU 1286) was deposited in the herbarium of the College of Pharmacy, Chungnam University.

Extraction and Isolation Dried plant material (6.0 kg) was extracted with hot MeOH, refluxed for 3 h (31×3 times) to yield a methanolic extract (320 g). The extract was sus-

* To whom correspondence should be addressed. e-mail: baekhh@cnu.ac.kr

pended in H₂O and then partitioned with CH₂Cl₂ and EtOAc. The aqueous layer was subjected to Diaion column chromatography and eluted with a stepwise gradient of H₂O and MeOH (H₂O→40% MeOH, 40→60% MeOH, and 60→100% MeOH) to give three subfractions (F1–F3). The subfraction F2 (35 g) was continually separated by MCI gel CHP20P column chromatography using H₂O and MeOH gradient (H₂O→30% MeOH, 35→80% MeOH) to give F2.1 (20 g) and F2.2 (6 g). F2.2 was re-subjected to MCI gel CHP20 column chromatography using 2% AcOH–MeOH (1:1), magnoflorine (15.5 mg) was obtained by crystallization in MeOH from the collected subfraction F2.2.3.

Magnoflorine: Colorless crystal, mp 210–212 °C; IR ν_{max} (KBr) 3400 (OH), 1645, 1640 (aromatic ring) cm⁻¹; UV, $\lambda_{\text{max}}^{\text{MeOH}}$ 230, 280, 323 nm; FAB-MS m/z 343 [M+H]⁺, C₂₀H₂₄NO₄. ¹H- and ¹³C-NMR spectral data were in accordance with published paper.^{23,24)}

HDL and LDL Preparation Blood from healthy normolipemic donors was obtained by venipuncture and collected in EDTA-containing vacutainer tubes. LDL was prepared from plasma by sequential flotation ultracentrifugation as described previously.²⁶⁾ To isolate HDL, plasma was prepared by centrifugation at 3000 rpm for 10 min and thereafter used for the preparation of plasma lipoproteins. HDL was isolated from plasma by ultracentrifugation for 1:30 h with a vertical rotor.²⁷⁾ After dialysis at 4 °C for 24 h against 10 mM phosphate-buffered saline (PBS) pH 7.4, HDL protein concentration (μg protein/ml) was determined as described by Lowry *et al.*²⁸⁾

Cu²⁺ Mediated HDL Oxidation The oxidation of HDL was assessed by the formation of conjugated dienes determined as the change in UV absorbance at 232 nm. Briefly, HDL (200 $\mu\text{g}/\text{ml}$) in PBS (pH 7.4) was pre-incubated with either the absence (control) or presence of magnoflorine (various concentrations), and then Cu²⁺ (5 μM) was added to initiate the oxidation at 37 °C. Absorbance at 232 nm was continuously monitored at 10 min intervals for 5 h at 37 °C using a spectrophotometer (Shimadzu UV-1240, Tokyo, Japan). The lag time was measured as the intercept between the baseline and the tangent of the absorbance curve during the propagation phase.^{29,30)} The oxidation of HDL to malondialdehyde (MDA) was measured using the thiobarbituric acid reactive substances (TBARS) assay. Briefly, HDL (200 $\mu\text{g}/\text{ml}$) in PBS (pH 7.4) was pre-incubated with magnoflorine, and then Cu²⁺ was added to initiate the oxidation. The reaction mixture was incubated at 37 °C for 2 h and the reaction was terminated by adding 20% trichloroacetic acid (TCA) and 1% thiobarbituric acid (TBA). After boiling at 95 °C for 15 min, the mixture was centrifuged at 10000 rpm for 10 min. The absorbance of supernatant was measured at 532 nm.

Peroxyl Radicals Mediated HDL Oxidation Oxidation of HDL by peroxyl radicals was performed by incubation of the HDL (200 $\mu\text{g}/\text{ml}$) at 37 °C in PBS (pH 7.4) with one of the thermo-labile radical initiator (AAPH) for 2 h. AAPH (10 mM) served as a source of hydrophilic peroxyl radicals.³¹⁾ At the end of the incubation, samples were also assessed by measuring accumulation of lipid aldehydes (TBARS) as described above.

Effect of Magnoflorine/Cu²⁺ Oxidized HDL in LDL Oxidation HDL (1000 $\mu\text{g}/\text{ml}$) was pre-incubated with Cu²⁺ (5 μM) for 1 h in the presence or absence of mag-

noflorine (0–10 μM) in PBS buffer (10 mM, pH 7.4) at 4 °C. Native HDL or oxidized HDL (0.1 mg/ml) was incubated with LDL (0.1 mg/ml) in the presence of Cu²⁺ (0.5 μM) at 37 °C, and 3 h later, the lipid oxidation was assessed by the measurement of peroxide values as described previously.³²⁾ The inhibition of LDL oxidation by HDL under these conditions was calculated according to the formular:

$$\text{inhibition of LDL oxidation (\%)} \\ = [(A_{\text{HDL}} + A_{\text{LDL}}) - A_{\text{HDL+LDL}}] \times 100 / (A_{\text{HDL}} + A_{\text{LDL}})$$

Where ($A_{\text{HDL}} + A_{\text{LDL}}$) is the sum of the absorbances observed for HDL and LDL incubated separately with Cu²⁺ for 3 h, and $A_{\text{HDL+LDL}}$ is the absorbance observed during co-incubation of LDL with HDL and Cu²⁺ for 3 h.

Statistical Analysis The results are expressed as mean values $\pm$ S.D. Statistical analysis was performed using one-way ANOVA. A $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Numerous studies have found that HDL is readily modified using a variety of oxidants *in vitro*. These include transition metal ions, aqueous peroxy and hydroxyl radicals, aldehydes, peroxidase-generated tyrosyl radical, cigarette smoke, lipoxygenase, hypochlorous acid, cultured endothelial cell, and macrophages.^{9,33)} As part of an ongoing study to search for antioxidants from traditional medicine plants, we have focused on the water layer of *Coptidis Rhizoma* with the expectation that we could find out more anti-lipid peroxidation agents. Repeated column chromatography led to the isolation of a colorless crystal compound. This compound was identified as magnoflorine by analyses of MS, NMR data, and comparison with those in the literature (Fig. 1).²³⁾

Since the formation of conjugated dienes represents the initiate phase of HDL oxidation, the extent of lag time is indicated as oxidation resistant capacity of HDL. As shown in Fig. 2, spectrophotometric analysis of Cu²⁺-induced HDL oxidation based on the conjugated dienes formation due to the unsaturated lipids is presented. When the HDL was incubated with Cu²⁺ alone, the lag time was 62 min, whereas, in the presence of magnoflorine (3.0 μM), the lag phase was retarded to 123 min. In this experiment, the lag phases of vitamin C (3.0 μM) and vitamin E (3.0 μM) retarded to 97 and 145 min, respectively (Table 1).

The oxidation of HDL initiated by Cu²⁺ and thermo-labile radical initiator AAPH was also measured by the formation of malondialdehyde (MDA) using the TBARS assay. As shown in Table 1, the compound tested remarkably reduced the formation of TBARS, comparable to the results of conjugated diene formation. Magnoflorine exhibited significant

Fig. 1. Magnoflorine Structure

Fig. 2. Conjugated Diene Effect of Magnoflorine on the Cu^{2+} -Mediated HDL Oxidation

HDL (200 $\mu\text{g}/\text{ml}$) in PBS (pH 7.4) was pre-incubated with vitamin C (3.0 μM), vitamin E (3.0 μM) or magnoflorine (3.0 μM), and then Cu^{2+} (5 μM) was added to initiate the oxidation at 37 $^{\circ}\text{C}$. Conjugated diene was measured by determined the absorbance at 232 nm continuously at 10 min intervals for 5 h at 37 $^{\circ}\text{C}$.

Table 1. Effect of Magnoflorine on the Oxidation of HDL by TBARS Method

Compound	Lag time (min) ^{a)}	TBARS, IC_{50} (μM) ^{b)}	
		Cu^{2+} -mediated	AAPH-mediated
Magnoflorine	123	$2.3 \pm 0.2^{*,\dagger}$	$6.2 \pm 0.5^{*,\dagger}$
Vitamin C ^{c)}	97	10.5 ± 0.8	18.7 ± 1.5
Vitamin E ^{c)}	145	1.8 ± 0.2	4.4 ± 0.5

a) The lag time of blank was estimated to be 52 min, b) the values represent mean $\pm$ S.D. of triplicate experiments, c) compound used as positive control; * $p < 0.05$ vs. vitamin C, $\dagger p < 0.05$ vs. vitamin E.

inhibitory activity against Cu^{2+} - and AAPH mediated HDL oxidation in the dose dependent manners entirely with IC_{50} values of 2.3 ± 0.2 and 6.2 ± 0.5 μM , respectively. Vitamin C showed inhibitory activity with IC_{50} values of 10.5 ± 0.8 μM and 18.7 ± 1.5 μM , meanwhile, vitamin E showed inhibitory activity with IC_{50} values of 1.8 ± 0.2 μM and 4.4 ± 0.5 μM , under Cu^{2+} and AAPH mediated oxidation.

The effective action of magnoflorine on the ability of HDL to protect LDL from Cu^{2+} (0.5 μM)-mediated oxidation was evaluated by the measurement of the peroxide value. As showed in Fig. 3, the oxidized HDL (HDL-0), which was prepared from exposure of HDL to Cu^{2+} (5 μM) in the absence of magnoflorine, expressed a slight protection (28.5%), while native HDL was highly effective (68.8%) in protecting LDL from the oxidation. The inclusion of magnoflorine/ Cu^{2+} oxidized HDL preparations (HDL-1.0, -2.0, -5.0, -10.0) decreased lipid oxidation of LDL progressively with increasing concentration of magnoflorine (1.0, 2.0, 5.0, 10.0 μM). It is suggested that the ability of magnoflorine/ Cu^{2+} oxidized HDL in preventing LDL oxidation maybe related to the oxidation state of HDL after pre-incubation.

Oxidative modifications that would affect the capability of HDL to protect against atherosclerosis are of considerable importance and interest. The exact beneficial effects of HDL *in vivo* remain unknown. HDL may also be protective by inhibiting the oxidation of LDL, and by neutralizing the atherogenic effects of oxidized LDL.⁹⁾ However, certain forms of oxidized HDL may actually enhance protection by stimulating the delivery of intracellular cholesterol to cell surface sites where it becomes available for removal by other (non-oxidized) HDL particles.¹⁰⁾ Lipid oxidation in HDL is promoted by a variety of factors. In this study, we used Cu^{2+} ,

Fig. 3. Effect of Magnoflorine/ Cu^{2+} Oxidized HDL in LDL Oxidation

After 3 h incubation, an aliquot was taken for the assay of lipid peroxidation, based on the measurement of the peroxide values. nHDL is native HDL. HDL-0, -1.0, -2.0, -5.0, and -10.0 indicate the HDL oxidized with Cu^{2+} in the presence of magnoflorine at 0, 1.0, 2.0, 5.0, and 10.0 μM , respectively. * $p < 0.05$ vs. nHDL, $\dagger p < 0.05$ vs. HDL-0.

a metal ion-independent oxidant, and AAPH, a hydrophilic peroxy radicals as the activation sources. That is consistent with those of numerous other investigators who reported that lipids in HDL are highly susceptible to oxidation during incubation with Cu^{2+} and AAPH.^{13,27,31)} Although there have been a number of reports on the designing and development of synthetic lipid peroxidation inhibitors, that were necessary for those other studies, which have been reported the HDL oxidation inhibitors derived from medicinal plants.^{27,30)} Specially, boldine, an antioxidant alkaloid isolated from *Peumus boldus*, which has similar chemical structure with magnoflorine, has been well established to have free radical scavenger, prevents the oxidation of LDL *in vitro* and *in vivo*.³⁴⁾

Our results clearly demonstrate that magnoflorine significantly inhibits lipid oxidation in HDL exposed to a metal ion-independent form and peroxy radicals mediated of oxidant stress. Magnoflorine, even at low concentration (3.0 μM) could exert a protective effect against Cu^{2+} induced lipid peroxidation of HDL, as showed by decrease the lag time of conjugated diene process. Interestingly, magnoflorine is more effective than vitamin C but less than vitamin E by retarding lag phase (Fig. 2), and further evidence by TBARS method (Table 1). Since magnoflorine was a more effective antioxidant in the metal-dependent pro-oxidant system (2.3 μM) compared to the peroxy radical system (6.2 μM), metal ion chelating properties may underlie magnoflorin's apparent antioxidant effects towards HDL oxidation *in vitro*. In addition, HDL has been recognized to have antioxidant action to protect LDL from oxidative modification, this might be supported by present finding that the oxidized HDL, prepared from the exposure of HDL to Cu^{2+} in combination with magnoflorine, exerted an antioxidant action against LDL oxidation.

The antioxidant effect of magnoflorine is due to its chemical structure. Magnoflorine is an alkaloid bearing two free phenolic groups. The presence of aromatic-OH group may be responsible for their antioxidant efficiency, similarly to phenolic antioxidants,³⁵⁾ *via* chain-breaking mechanism by donation of phenolic hydrogen. Moreover, magnoflorine showed a lower value of O-H bond dissociation energy, and highest occupied molecular orbital surroundings of the reaction center, which have been identified as important requisites for both chelating and radical scavenging activity and explaining the higher antioxidant efficiency of the former compound.²⁵⁾

The hypothesis that Coptidis Rhizoma would be useful as an alternative therapy for hypercholesterolemia, thus, mag-

noflorine and some other constituents are able to play an important role on reducing oxidative stress, prevent the development and progression of atherosclerotic disease. In summary, our results demonstrate that magnoflorine could inhibit lipid oxidation in HDL but the cardioprotective ability of this lipoprotein fraction in combination with magnoflorine to prevent atherogenic modification of LDL should be more preserved.

Acknowledgements This work was supported by a grant funded from the Korean Food and Drug Administration (2007).

REFERENCES

- 1) National Heart, Lung, and Blood Institute, Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III): Final Report, National Institutes of Health (2002).
- 2) Nofer J. R., Kehrel B., Fobker M., Levkau B., Assmann G., von Eckardstein A., *Atherosclerosis*, **161**, 1—16 (2002).
- 3) Assmann G., Nofer J. R., *Annu. Rev. Med.*, **54**, 321—341 (2003).
- 4) Stampfer M. J., Sacks M. D., Salvini S., Willett S. C., Hennekens C. H., *New Engl. J. Med.*, **325**, 373—381 (1991).
- 5) Rubins H. B., Robins S. J., Iwane M. K., Boden W. E., Elam M. B., Fye C. L., Gordon D. J., Schaefer E. J., Schectman G., Wittes J. T., *Am. J. Cardiol.*, **71**, 45—52 (1993).
- 6) Rubin E. M., Krauss R. M., Spangler E. A., Verstuyft J. G., Clift S. M., *Nature* (London), **353**, 265—267 (1991).
- 7) Paszty C., Maeda N., Verstuyft J., Rubin E. M., *J. Clin. Invest.*, **94**, 899—903 (1994).
- 8) Plump A. S., Scott C. J., Breslow J. L., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 9607—9611 (1994).
- 9) Francis G. A., *Biochim. Biophys. Acta*, **1483**, 217—235 (2000).
- 10) Nagano Y., Arai H., Kita T., *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 6457—6461 (1991).
- 11) Parthasarathy S., Barnett J., Fong L. G., *Biochim. Biophys. Acta*, **1044**, 275—283 (1990).
- 12) Jaouad L., Miloshevitich C., Khalil A., *Free Radic. Res.*, **37**, 77—83 (2003).
- 13) Hillstrom R. J., Yacopin-Ammons A. K., Lynch S. M., *J. Nutr.*, **133**, 3047—3051 (2003).
- 14) Iizuka N., Miyamoto K., Hazama S., Yoshino S., Yoshimura K., Okita K., Fukumoto T., Yamamoto S., Tangoku A., Oka M., *Cancer Lett.*, **158**, 35—41 (2000).
- 15) Lee D. U., Kang Y. J., Park M. K., Lee Y. S., Seo H. G., Kim T. S., Kim C. H., Chang K. C., *Life Sci.*, **73**, 1401—1412 (2003).
- 16) Hwang B. Y., Roberts S. K., Chadwick L. R., Wu C. D., Kinghorn A. D., *Planta Med.*, **69**, 623—627 (2003).
- 17) Yu H. H., Kim K. J., Cha J. D., Kim H. K., Lee Y. E., Choi N. Y., You Y. O., *J. Med. Food*, **8**, 454—461 (2005).
- 18) Higaki S., Nakamura M., Morohashi M., Hasegawa Y., Yamagishi T., *J. Dermatol.*, **23**, 871—875 (1996).
- 19) Nam C., Kim S., Sim Y., Chang I., *Skin Pharmacol. Appl. Skin Physiol.*, **16**, 84—90 (2003).
- 20) Yokozawa T., Ishida A., Cho E. J., Nakagawa T., *Phytomedicine*, **10**, 17—22 (2003).
- 21) Otsuka H., Tsukui M., Matsuoka T., Goto M., Fujimura H., Hiramatsu Y., Sawada T., *Yakugaku Zasshi*, **94**, 796—801 (1974).
- 22) Peng W. H., Haieh M. T., Wu C. R., *Jpn. J. Pharmacol.*, **74**, 261—266 (1997).
- 23) Mori H., Fuchigami M., Inoue N., Nagai H., Koda A., Nishioka I., *Planta Med.*, **60**, 445—449 (1994).
- 24) Min Y. D., Choi S. U., Lee K. R., *Arch. Pharm. Res.*, **29**, 627—632 (2006).
- 25) Račková L., Májeková M., Košťálová D., Štefek M., *Bioorg. Med. Chem.*, **12**, 4709—4715 (2004).
- 26) Hung T. M., Na M. K., Thuong P. T., Su. D., Sok D. E., Song K. S., Seong Y. H., Bae K., *J. Ethnopharmacol.*, **108**, 188—192 (2006).
- 27) Ferretti G., Bacchetti T., Menanno F., Curatola G., *Atherosclerosis*, **172**, 55—61 (2004).
- 28) Lowry O. H., Rosebrough N. J., Farr A. L., Randall P. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
- 29) Puhl H., Waeg G., Esterbauer H., *Methods Enzymol.*, **233**, 425—441 (1994).
- 30) Sobal G., Sinzinger H., *Biochem. Pharmacol.*, **70**, 1185—1191 (2005).
- 31) Calla M. S., Lynch S. M., *Arch. Biochem. Biophys.*, **452**, 129—137 (2006).
- 32) Raveh O., Pinchuk I., Schnitzer E., Fainaru M., Schaffer Z., Lichtenberg D., *Free Radic. Biol. Med.*, **29**, 131—146 (2000).
- 33) Steinberg D., *J. Biol. Chem.*, **272**, 20963—20966 (1997).
- 34) Santanam N., Penumetcha M., Speisky H., Parthasarathy S., *Atherosclerosis*, **173**, 203—210 (2004).
- 35) Bors W., Heller W., Michel C., Saran M., *Methods Enzymol.*, **186**, 343—355 (1990).