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Selenoprotein P Protects Low-density Lipoprotein Against Oxidation

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Selenoprotein P (SeP) is an extracellular glycoprotein with 8–10 selenocysteines per molecule, containing approximately 50% of total selenium in human serum. An antioxidant function of SeP has been postulated. In the present study, we show that SeP protects low-density lipoproteins (LDL) against oxidation in a cell-free *in-vitro* system. LDL were isolated from human blood plasma and oxidized with $CuCl_2$, 2,2′-azobis(2-amidinopropane) (AAPH) or peroxynitrite in the presence or absence of SeP, using the formation of conjugated dienes as parameter for lipid peroxidation. SeP delayed the $CuCl_2$- and AAPH-induced LDL oxidation significantly and more efficiently than bovine serum albumin used as control. In contrast, SeP was not capable of inhibiting peroxynitrite-induced LDL oxidation. The protection of LDL against $CuCl_2$- and AAPH-induced oxidation provides evidence for the antioxidant capacity of SeP. Because SeP associates with endothelial membranes, it may act *in vivo* as a protective factor inhibiting the oxidation of LDL by reactive oxygen species.

Keywords: Selenium; Selenoprotein P; Low-density lipoprotein; Oxidation; Peroxynitrite

INTRODUCTION

The essential trace element selenium exerts many of its biological effects as a constituent of selenoproteins, containing selenocysteine (Sec) in their polypeptide chain. Recently, the human selenoproteome was reported to consist of 25 selenoproteins, based on a computational analysis of genome sequences.[1] Selenoprotein P (SeP) is unique among all selenoproteins, containing two Sec-insertion sequence (SECIS) elements and 10 UGA codons in its mRNA sequence. This predicts for the presence of up to 10 Sec residues in the amino acid sequence of SeP, while all other known selenoproteins possess only one Sec.[2] SeP is a highly glycosylated plasma protein, produced and secreted mainly by the liver, but its expression was detected in various other organs including heart, kidney and brain.[3,4] SeP is the major selenoprotein in human blood plasma,[5] where the SeP protein level has been estimated to be 40 nM.[6] The biological role of SeP has yet to be fully elucidated. Currently, there is evidence for three functions: SeP has been postulated to be involved in the protection of cellular membranes against oxidative damage,[3] to sequester cadmium and mercury ions[7] and to transport selenium from the liver to various other organs including the brain.[8,9] While a selenium transport function of SeP was recently supported by two studies using SeP knockout mice,[10,11] there are several reports on antioxidant properties of SeP. In rats, SeP protected from oxidative damage of the liver induced by diquat.[12] Furthermore, SeP was shown to reduce phospholipid hydroperoxide with glutathione or thioredoxin as electron donors in a cell-free *in-vitro* system.[13,14] Our group reported that SeP in human plasma protects against peroxynitrite-mediated oxidation and nitration.[15]

Low-density lipoprotein (LDL), circulating in blood plasma, supply various cells with cholesterol under normal physiological conditions, but oxidized LDL have cytotoxic effects and are thought to be involved in the development of atherosclerosis.[16] As SeP is capable of reducing phospholipid

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hydroperoxides[13] and to bind glycosaminoglycans,[17] we hypothesized that SeP may take part in the protection of LDL against oxidation upon binding to apolipoprotein B-100 (apo B), a glycosylated LDL component. In the present study, we show that SeP, purified from human blood plasma, efficiently protects LDL against oxidation by copper(II) chloride or 2,2′-azobis(2-amidinopropane) (AAPH) in a cell-free *in-vitro* system. Our data suggest that SeP can be a protective factor delaying the formation of oxidized LDL *in vivo*, of interest in the prevention of atherosclerosis.

MATERIALS AND METHODS

Reagents

Reagents for SDS-polyacrylamide gel electrophoresis were purchased from Roth (Karlsruhe, Germany). If not stated otherwise, all other reagents were obtained from Sigma (Taufkirchen, Germany). The polyclonal primary antibody against human SeP was produced in rabbits as described,[18] while the secondary anti-rabbit IgG- horseradish peroxidase (HRP)-coupled antibody was obtained from Dianova (Hamburg, Germany). Peroxynitrite was synthesized from sodium nitrite and H_2O_2 as described[19] and its concentration was determined spectrophotometrically at 302 nm ($\varepsilon = 1670\,M^{-1} \times cm^{-1}$).

Preparation of SeP

SeP was purified from human blood plasma as described.[18] Plasma samples were generously provided by the blood bank of Heinrich-Heine-Universität Düsseldorf. All solutions used for the SeP preparation contained 1 mM phenylmethylsulfonylfluoride (PMSF) as protease inhibitor. Briefly, the preparation of SeP was carried out by three successive chromatography steps: the heparin-binding proteins were isolated from plasma by affinity chromatography using heparin-sepharose CL-6B (Amersham Pharmacia, Freiburg, Germany), which was followed by ion exchange chromatography on Q-sepharose fast flow (Amersham Pharmacia) and another affinity chromatography using Ni-NTA superflow (Qiagen, Hilden, Germany). Aliquots of all fractions of the Ni-NTA affinity chromatography were tested by Western blotting and silver staining of SDS-PAGE gels. The aliquots containing purified SeP were pooled and dialysed once against water and once against phosphate-buffered saline (PBS). The protein concentrations of the SeP samples were then determined by a Bradford protein assay (Biorad, München, Germany).

Preparation of LDL

LDL were isolated from blood plasma by density gradient centrifugation as described.[20] Centrifugation was carried out in a L8-60M ultracentrifuge (Beckman, Palo Alto, CA) with an SW 41 Ti rotor for 2 h at 120,000*g* and 15°C. The LDL were enriched upon centrifugation in the intermediate phase between a high density (354 g/l KBr, 153 g/l NaCl, 0.1 mg/l EDTA) and a low density (8.7 g/l NaCl) solution. Thereafter, the purified LDL were desalted on a Sephadex PD10 column (Amersham Pharmacia) and its concentration was determined by measuring the cholesterol content of the solution with the CHOD-PAP-Kit (Roche Diagnostics, Mannheim, Germany) and a cholesterol standard (Sigma). The constant cholesterol content of LDL of 31.6%[21] was used to calculate the LDL concentration in solution.

SeP Immunodetection in Western and Dot Blots

The proteins were separated by SDS-PAGE with 12% polyacrylamide separating gels and 4% polyacrylamide stacking gels under denaturing conditions according to the method of Laemmli.[22] For Western blots, proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia, Freiburg, Germany) after gel electrophoresis. For Dot blots, 1 μl of protein solution was spotted onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia). Immunodetection was carried out using a polyclonal SeP primary antibody[18] and a HRP-conjugated goat-anti rabbit IgG secondary antibody (Dianova, Hamburg, Germany). SeP was detected by a chemiluminescence system (ECL Plus, Amersham Pharmacia) on Biomax light film (Kodak, Rochester, NY).

Oxidation of LDL

Purified LDL were oxidized at 37°C in 1.5 ml quartz cuvettes containing 1 ml PBS, 0.1 μM LDL, oxidant and SeP or bovine serum albumin (BSA). LDL oxidation was carried out with $CuCl_2$ (10 μM), AAPH (1 mM) or peroxynitrite, which was infused from a 10 mM stock solution with a micropump to yield a steady-state concentration of 7 nM as described.[15] The time course of LDL oxidation was followed by measuring the absorbance of the solution at 234 nm with a Lambda 2 spectrophotometer (Perkin Elmer, Boston, MA). As conjugated dienes with an absorbance maximum at 234 nm are formed as typical products during the oxidation of unsaturated fatty acids or LDL, its measurement is used as standard method for the determination of LDL oxidation.[23]

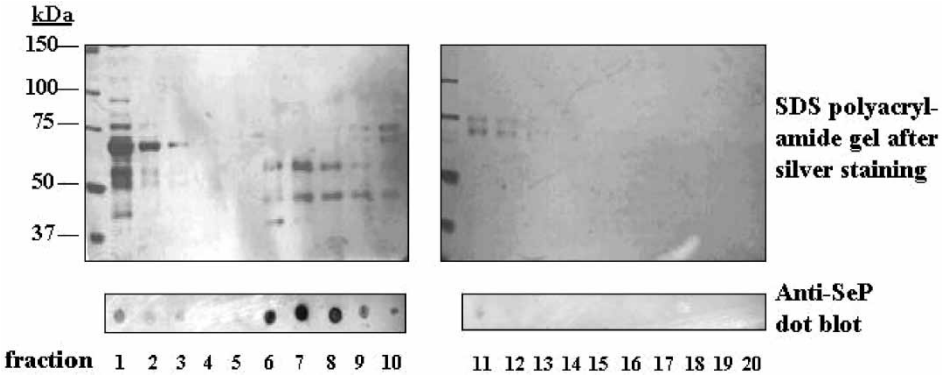

FIGURE 1 Elution profile of plasma proteins after Ni^{2+}-NTA-chromatography. Aliquots of the protein fractions of the Ni^{2+}-NTA-chromatography were separated on a 12% SDS polyacrylamide gel, and proteins were detected by silver staining. In parallel, other aliquots were subjected to dot blot, and SeP was detected by immunostaining with a polyclonal rabbit anti-SeP antibody.

RESULTS

Preparation of Purified SeP from Human Blood Plasma

SeP was purified from human plasma by three successive chromatography steps according to the method of Mostert *et al.*[18] Aliquots of all fractions obtained after the Ni-NTA affinity chromatography were analyzed by silver staining of SDS polyacrylamide gels and SeP immunodetection of dot blots. Fractions 6–8 showed the highest immunoreactivity against an anti-SeP antibody in the dot blot and yielded a pure preparation of SeP consisting of two isoformes with a molecular mass of 61 and 51 kDa, respectively (Fig. 1). Therefore, these fractions were pooled and used for further experiments.

Effect of SeP on $CuCl_2$- and AAPH-induced Oxidation of LDL

LDL purified from human plasma were oxidized by $CuCl_2$, and the effect of SeP and bovine serum albumin (BSA) on the oxidation process was investigated. SeP and BSA have a similar molecular mass and were used at the same concentration (260 nM). As shown in Fig. 2, $CuCl_2$ caused an oxidation of LDL, detected by spectrophotometric measurement of conjugated dienes at 234 nm. The $CuCl_2$-mediated formation of conjugated dienes started after a lag period of 40 min upon addition of $CuCl_2$ to the LDL solution. In the presence of 260 nM SeP, $CuCl_2$-induced oxidation was effectively delayed, so that the lag period was extended by 50% to 60 min. By contrast, 260 nM BSA had only

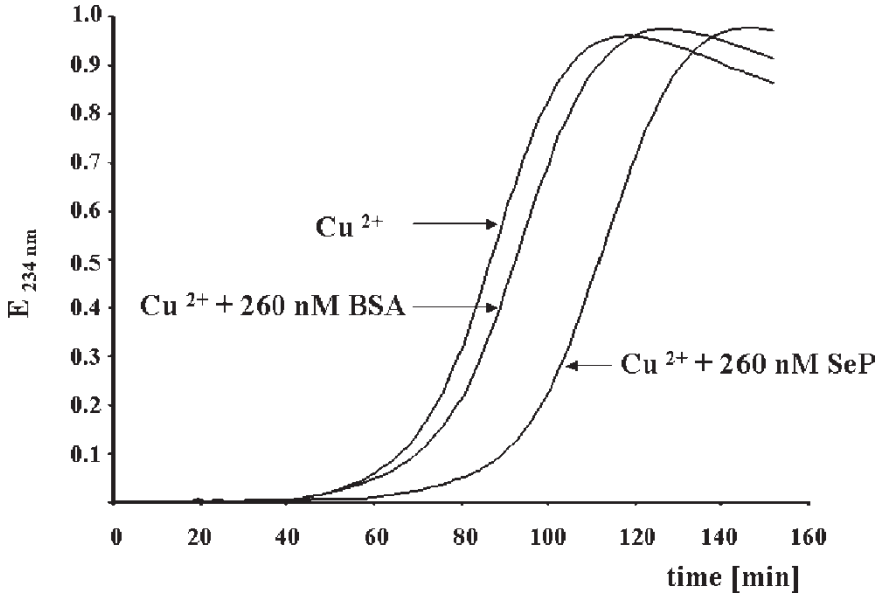

FIGURE 2 Effect of SeP on $CuCl_2$-induced LDL oxidation. Purified LDL (0.1 μM) in PBS were oxidized with $CuCl_2$ (10 μM) at 37°C. The LDL were incubated with $CuCl_2$ alone or in the presence of 260 nM SeP or 260 nM BSA, respectively. LDL oxidation was determined by spectrophotometric measurement of the formation of conjugated dienes at 234 nm. The figure is representative of three independent experiments with similar results.

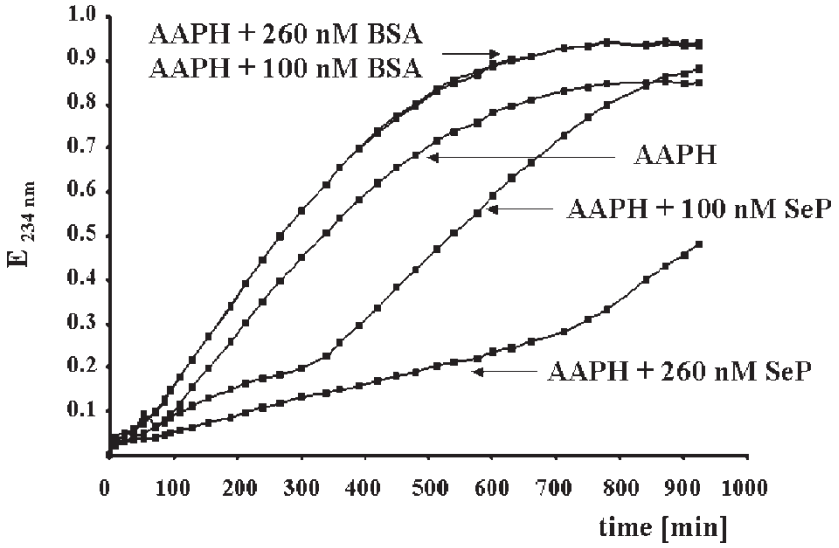

FIGURE 3 Effect of SeP on AAPH-induced LDL oxidation. Purified LDL (0.1 μM) in PBS were oxidized with AAPH (1 mM) at 37°C. The LDL were incubated with AAPH alone or pre-incubated with SeP (100 or 260 nM) or BSA (100 or 260 nM), respectively. LDL oxidation was determined as in Fig. 2. The figure is representative of two (260 nM) or three (100 nM) independent experiments with similar results.

a very slight inhibitory effect, implying that the attenuation of LDL oxidation by SeP is not a mere protein effect. Even a lower concentration of SeP (100 nM) exhibited a stronger protective effect against $CuCl_2$-induced LDL oxidation than 260 nM BSA (data not shown).

In order to exclude simple Cu^{2+} binding as the mechanism for protection by SeP, another inducer of LDL oxidation was employed. Purified LDL were oxidized with AAPH, which decomposes to N_2 and alkyl radicals (R·). Alkyl radicals react with O_2 to form peroxyl radicals (R–OO·), capable of initiating lipid peroxidation.[24] As shown in Fig. 3, AAPH oxidized LDL, but the oxidation process was much slower than with $CuCl_2$. The formation of conjugated dienes upon AAPH addition was therefore measured for a total time of 1000 min. SeP protected LDL against oxidation with AAPH, delaying the oxidation process dependent on its concentration. By contrast, BSA did not protect LDL against AAPH-induced oxidation.

Effect of SeP on Peroxynitrite-induced Oxidation of SeP

SeP is known to protect against peroxynitrite-induced benzoate hydroxylation and nitration of human plasma proteins.[15] Therefore, we tested whether SeP would protect against peroxynitrite-induced LDL oxidation. LDL were oxidized by steady-state infusion of peroxynitrite, which led to a linear increase in absorbance at 234 nm without an initial lag period (Fig. 4A). However, SeP had only a slight protective effect against peroxynitrite-induced LDL oxidation, which was non-specific for SeP, as BSA protected equally well (Fig. 4B).

DISCUSSION

In this report, we demonstrate that SeP, purified from human blood plasma, can protect LDL against oxidation in a cell-free *in-vitro* system. SeP significantly delayed the $CuCl_2$- or AAPH-induced formation of conjugated dienes.

Copper (II) ions cause lipid peroxidation upon binding to LDL,[24] which leads to reduction of Cu^{2+} to Cu^{1+} subsequently initiating the oxidation reaction by catalysing the formation of radicals.[25] Schnitzer *et al.*[26] reported that bovine serum albumin (BSA) has a slight protective effect against $CuCl_2$-mediated LDL oxidation, mainly by binding Cu^{2+} ions. Comparing the antioxidant potential of BSA and SeP, SeP was more effective even when used at lower concentration than BSA. As both proteins have a similar molecular mass, the protective function of SeP is unlikely to be due to an unspecific protein effect. SeP could delay the LDL oxidation by scavenging of radicals, reduction of hydroperoxides[13,14] or by protecting endogenous antioxidants like vitamin E. LDL contain apo B-100,[27] a glycosylated protein which could serve as binding site for SeP, because SeP binds to glycosaminoglycans.[17]

SeP also had a protective effect against the AAPH-induced oxidation of LDL, delaying the formation of conjugated dienes by several hours. AAPH causes a slower oxidation than $CuCl_2$ upon its decomposition, leading to the formation of peroxyl radicals (R–OO·).[24] We hypothesize that SeP exerts its antioxidant function by direct interaction with these radicals. In contrast to SeP, BSA showed no protective effect against AAPH-induced LDL oxidation, in accordance with a previous

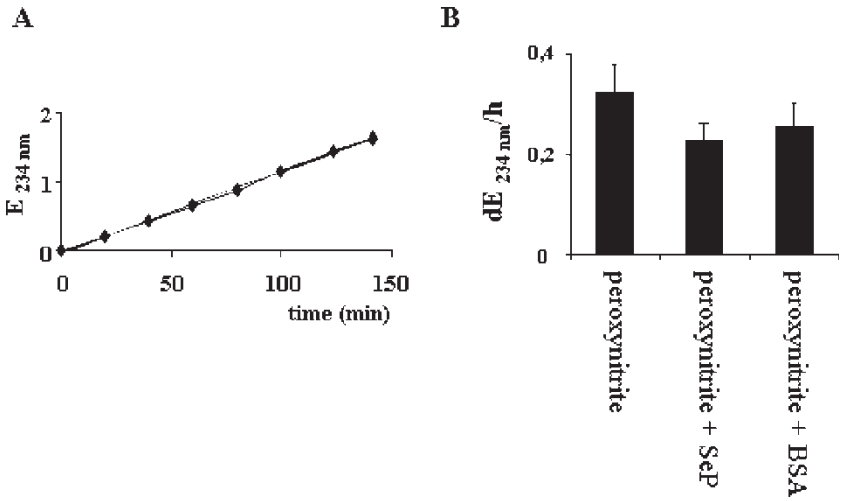

FIGURE 4 Effect of SeP on peroxynitrite-induced LDL oxidation. Purified LDL (0.1 μM) in PBS were oxidized with peroxynitrite at 37°C by infusion with a micropump to maintain a steady-state concentration of 7 nM peroxynitrite. The LDL were incubated with peroxynitrite alone or pre-incubated with 100 nM SeP and LDL oxidation was determined for 150 min as in Fig. 2. The figure is representative of three independent experiments with similar results (A). LDL were incubated with peroxynitrite alone or pre-incubated with 100 nM SeP or 100 nM BSA, respectively, and the increase of absorbance at 234 nm per hour was determined. The data represent mean values ± SD from three independent experiments (B).

study,[26] supporting our hypothesis of SeP as a radical scavenger.

Our group has shown before that SeP in human blood plasma efficiently protects against peroxynitrite-induced oxidation and nitration.[15] However, in contrast to this previous study, the peroxynitrite-induced oxidation of LDL was only slightly delayed by SeP. This slight protection was rather unspecific, because BSA showed a similar effect. These results imply that $CuCl_2$, AAPH (generation of one-electron oxidants) and peroxynitrite (two-electron oxidant) may oxidize LDL by different mechanisms. SeP may provide effective protection only when LDL are oxidized by one-electron oxidants. A similar effect was described for vitamin E: α-tocopherol only prevents lipid peroxidation by one-electron oxidants.[28] Two-electron oxidants such as hypochloric acid oxidize LDL by a different mechanism, and α-tocopherol has only a slight protective effect.[29]

As SeP protects LDL against oxidation and reduces lipid peroxides *in vitro*,[13,14] it is tempting to speculate that it might have an anti-atherogenic function, preventing the generation of oxidized LDL *in vivo*. In fact, low serum selenium levels have been reported to be correlated with a higher risk for ischemic heart disease and carotid atherosclerosis in clinical studies.[30,31] Therefore, a lowered SeP level in the serum could be a possible risk factor for atherogenesis.

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References

[1] Kryukov, G.V., Castellano, S., Novoselov, S.V., Lobanov, A.V., Zehtab, O., Guigo, R. and Gladyshev, V.N. (2003) "Characterization of mammalian selenoproteomes", *Science* **300**, 1439–1443.
[2] Burk, R.F. and Hill, K.E. (1999) "Orphan selenoproteins", *Bioessays* **21**, 231–237.
[3] Burk, R.F. and Hill, K.E. (1994) "Selenoprotein P. A selenium-rich extracellular glycoprotein", *J. Nutr.* **124**, 1891–1897.
[4] Dreher, I., Schmutzler, C., Jakob, F. and Köhrle, J. (1997) "Expression of selenoproteins in various rat and human tissues and cell lines", *J. Trace Elem. Med. Biol.* **11**, 83–91.
[5] Burk, R.F., Hill, K.E. and Motley, A.K. (2001) "Plasma selenium in specific and non-specific forms", *Biofactors* **14**, 107–114.
[6] Akesson, B., Bellew, T. and Burk, R.F. (1994) "Purification of selenoprotein P from human plasma", *Biochim. Biophys. Acta* **1204**, 243–249.
[7] Yoneda, S. and Suzuki, K.T. (1997) "Equimolar Hg–Se complex binds to selenoprotein P", *Biochem. Biophys. Res. Commun.* **231**, 7–11.
[8] Motsenbocker, M.A. and Tappel, A.L. (1982) "A selenocysteine-containing selenium-transport protein in rat plasma", *Biochim. Biophys. Acta* **719**, 147–1453.
[9] Burk, R.F., Hill, K.E., Read, R. and Bellew, T. (1991) "Response of rat selenoprotein P to selenium administration and fate of its selenium", *Am. J. Physiol.* **261**, E26–E30.
[10] Hill, K.E., Zhou, J., McMahan, W.J., Motley, A.K., Atkins, J.F., Gesteland, R.F. and Burk, R.F. (2003) "Deletion of selenoprotein P alters distribution of selenium in the mouse", *J. Biol. Chem.* **278**, 13640–13646.
[11] Schomburg, L., Schweizer, U., Holtmann, B., Flohé, L., Sendtner, M. and Köhrle, J. (2003) "Gene disruption discloses role of selenoprotein P in selenium delivery to target tissues", *Biochem. J.* **370**, 397–402.
[12] Burk, R.F., Hill, K.E., Awad, J.A., Morrow, J.D., Kato, T., Cockell, K.A. and Lyons, P.R. (1995) "Pathogenesis of diquat-induced liver necrosis in selenium-deficient rats: assessment of the roles of lipid peroxidation and selenoprotein P", *Hepatology* **21**, 561–569.

[13] Saito, Y., Hayashi, T., Tanaka, A., Watanabe, Y., Suzuki, M., Saito, E. and Takahashi, K. (1999) "Selenoprotein P in human plasma as an extracellular phospholipid hydroperoxide glutathione peroxidase. Isolation and enzymatic characterization of human selenoprotein P", *J. Biol. Chem.* **274**, 2866–2871.
[14] Takebe, G., Yarimizu, J., Saito, Y., Hayashi, T., Nakamura, H., Yodoi, J., Nagasawa, S. and Takahashi, K. (2002) "A comparative study on the hydroperoxide and thiol specificity of the glutathione peroxidase family and selenoprotein P", *J. Biol. Chem.* **277**, 41254–41258.
[15] Arteel, G.E., Mostert, V., Oubrahim, H., Briviba, K., Abel, J. and Sies, H. (1998) "Protection by selenoprotein P in human plasma against peroxynitrite-mediated oxidation and nitration", *Biol. Chem.* **379**, 1201–1205.
[16] Berliner, J.A. and Heinecke, J.W. (1996) "The role of oxidized lipoproteins in atherogenesis", *Free Radic. Biol. Med.* **20**, 707–727.
[17] Arteel, G.E., Franken, S., Kappler, J. and Sies, H. (2000) "Binding of selenoprotein P to heparin: characterization with surface plasmon resonance", *Biol. Chem.* **381**, 265–268.
[18] Mostert, V., Lombeck, I. and Abel, J. (1998) "A novel method for the purification of selenoprotein P from human plasma", *Arch. Biochem. Biophys.* **357**, 326–330.
[19] Koppenol, W.H., Kissner, R. and Beckman, J.S. (1996) "Syntheses of peroxynitrite: to go with the flow or on solid grounds?", *Methods Enzymol.* **269**, 296–302.
[20] Kleinveld, H.A., Hak-Lemmers, H.L., Stalenhoef, A.F. and Demacker, P.N. (1992) "Improved measurement of low-density-lipoprotein susceptibility to copper-induced oxidation: application of a short procedure for isolating low-density lipoprotein", *Clin. Chem.* **38**, 2066–2072.
[21] Pühl, H., Waeg, G. and Esterbauer, H. (1994) "Methods to determine oxidation of low-density lipoproteins", *Methods Enzymol.* **233**, 425–441.
[22] Laemmli, U.K. (1970) "Cleavage of structural proteins during the assembly of the head of bacteriophage T4", *Nature* **227**, 680–685.
[23] Esterbauer, H., Striegl, G., Pühl, H. and Rotheneder, M. (1989) "Continuous monitoring of *in vitro* oxidation of human low density lipoprotein", *Free Radic. Res. Commun.* **6**, 67–75.
[24] Thomas, M.J., Chen, Q., Franklin, C. and Rudel, L.L. (1997) "A comparison of the kinetics of low-density lipoprotein oxidation initiated by copper or by azobis(2-amidinopropane)", *Free Radic. Biol. Med.* **23**, 927–935.
[25] Lass, A., Witting, P., Stocker, R. and Esterbauer, H. (1996) "Inhibition of copper- and peroxyl radical-induced LDL lipid oxidation by ebselen: antioxidant actions in addition to hydroperoxide-reducing activity", *Biochim. Biophys. Acta* **1303**, 111–118.
[26] Schnitzer, E., Pinchuk, I., Bor, A., Fainaru, M. and Lichtenberg, D. (1997) "The effect of albumin on copper-induced LDL oxidation", *Biochim. Biophys. Acta* **1344**, 300–311.
[27] Esterbauer, H., Gebicki, J., Pühl, H. and Jürgens, G. (1992) "The role of lipid peroxidation and antioxidants in oxidative modification of LDL", *Free Radic. Biol. Med.* **13**, 341–390.
[28] Keaney, J.F. and Vita, J.A. (2002) "Vascular oxidative stress and antioxidant protection in atherosclerosis: what do the clinical trials say?", *J. Cardiopulm. Rehabil.* **22**, 225–233.
[29] Hazell, L.J. and Stocker, R. (1997) "Alpha-tocopherol does not inhibit hypochlorite- induced oxidation of apolipoprotein B-100 of low-density lipoprotein", *FEBS Lett.* **414**, 541–544.
[30] Suadicani, P., Hein, H.O. and Gyntelberg, F. (1992) "Serum selenium concentration and risk of ischaemic heart disease in a prospective cohort study of 3000 males", *Atherosclerosis* **96**, 33–42.
[31] Salonen, J.T., Salonen, R., Seppanen, K., Kantola, M., Suntioinen, S. and Korpela, H. (1991) "Interactions of serum copper, selenium, and low density lipoprotein cholesterol in atherogenesis", *BMJ* **302**, 756–760.