Antioxidant Activities of Chitobiose and Chitotriose

An-Shu CHEN,[a] Tadao TAGUCHI,[a] Kazuo SAKAI,[b] Kazuaki KIKUCHI,[b] Min-Wei WANG,[c] and Ichitomo MIWA*,[a]

[a] *Department of Pathobiochemistry, Faculty of Pharmacy, Meijo University; Tempaku-ku, Nagoya 468–8503, Japan:* [b] *Yaizu Suisankagaku Industry Co.; Yaizu 425–8570, Japan: and* [c] *Department of Pharmacology, School of Pharmacy, Shenyang Pharmaceutical University; Shenyang 110016, China.* Received February 5, 2003; accepted June 4, 2003

Chitooligosaccharides, the oligomers made up of β-1,4-linked D-glucosamine, are obtained by partial hydrolysis of chitosan, a deacetylation product of chitin. The antioxidant activity of various chitooligosaccharides was tested *in vitro* with aminoguanidine, pyridoxamine, and Trolox as reference compounds. Hydroxylation of benzoate to salicylate by H_2O_2 in the presence of Cu^{2+} was effectively inhibited by chitobiose, chitotriose, aminoguanidine, pyridoxamine, and Trolox (their IC_{50} values=18, 80, 85, 10, and 95 μM, respectively), whereas glucosamine and *N*-acetylchito-oligosaccharides (di-*N*-acetylchitobiose and tri-*N*-acetylchitotriose) did not show any inhibitory activity. Chitobiose and chitotriose were more potent than the 3 reference compounds in scavenging hydroxyl radicals produced by photolysis of zinc oxide: IC_{50} values of the 2 oligomers were 30 and 55 μM, respectively. Such a scavenging activity of these 2 chitooligomers was also shown by the use of another system, a mixture of Fe^{3+}/EDTA/ascorbate/H_2O_2, for producing hydroxyl radicals. Only chitobiose and Trolox, of the 10 compounds tested, had the ability to scavenge superoxide radicals generated by a non-enzymatic system using phenazine methosulfate and NADH. Taken together with our unpublished observation that chitobiose and chitotriose are appreciably absorbed from the intestine of rats, the present results suggest that these 2 chitooligosaccharides would act as effective antioxidants *in vivo* when orally ingested.

Key words chitobiose; chitotriose; antioxidant activity; chitooligosaccharide; aminoguanidine; pyridoxamine

Chitosan, a polysaccharide made up of β-1,4-linked D-glucosamine residues, is produced by deacetylation of chitin obtained from crab and prawn shells. This polysaccharide is not specifically hydrolyzed by mammalian digestive enzymes, and only limited hydrolysis may occur by the action of enzymes produced by bacterial flora. In recent years, chitosan has attracted much attention as a new biomedical material owing to its unique antibacterial,[1,2] hypoglycemic,[3—5] wound-healing,[6,7] and hypocholesterolemic[8—10] activities as well as others.[11] Even though chitosan has various biologically important properties, its high molecular weight and insolubility in the neutral pH region may restrict its use *in vivo*. In addition, although the toxicity of chitosan is known to be low,[11] some adverse effects have been reported, including excessive excretion of essential fatty acids[12] and decreased absorption of fat-soluble vitamins and minerals.[13]

We consider that it is worthwhile to study the functional properties of the oligosaccharides made from chitosan, because these oligosaccharides, as distinct from chitosan itself, have lower viscosity and are soluble in neutral aqueous solutions. In addition, their toxicity is very low.[14] Mixtures of chitooligosaccharides have been reported to have antibacterial,[1,15] antifungal,[16] antioxidant,[17] antimutagenic,[18] and leukemia cell differentiation-inducing activities.[19] Suzuki *et al.* reported that chitohexaose showed antitumor activity.[20,21] The biological activities of each of the chitooligosaccharides, however, remain to be studied further.

In the present paper, the antioxidant activities of several chitooligosaccharides, especially chitobiose and chitotriose, were studied in different *in vitro* systems.

MATERIALS AND METHODS

Materials Phenazine methosulfate, nitro blue tetrazolium, pyridoxamine dihydrochloride, D-glucosamine hydrochloride, Trolox (2-carboxy-2,5,7,8-tetramethyl-6-chromanol, a water-soluble α-tocopherol analogue), aminoguanidine hydrochloride, and horseradish peroxidase (Type XII) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). A series of chitooligosaccharide hydrochlorides, di-*N*-acetylchitobiose, and tri-*N*-acetylchitotriose were purchased from Seikagaku (Tokyo, Japan). Chelex-100 was obtained from Nippon Bio-Rad (Yokohama, Japan).

Inhibition of H_2O_2-Induced Hydroxylation of Benzoate Hydroxylation of benzoate by H_2O_2 was measured by the method of Giardino *et al.*[22] except that $CuSO_4$ was supplemented to the reaction mixture. Briefly, 100 mM sodium phosphate buffer (pH 7.4) containing 30 mM sodium benzoate, 10 mM H_2O_2, and 0.1 μM $CuSO_4$ was incubated for 16 h at 37 °C in the presence and absence of a test compound. Then the amount of salicylate formed by the reaction was determined by HPLC with a TSK gel ODS-80TM column (150×4.6 mm, 5 μm; Tosoh, Tokyo, Japan). The mobile phase was 10% (v/v) acetonitrile containing 20 mM potassium dihydrogen phosphate, and chromatography was performed at a flow rate of 1.0 ml/min and at a column temperature of 37 °C. Salicylate was monitored by measuring fluorescence at excitation and emission wavelengths of 308 and 410 nm, respectively.

Scavenging of Hydroxyl Radicals Produced by Photolysis of Zinc Oxide The hydroxyl radical-scavenging activities of test compounds were determined by the method of Russell *et al.*[23] except that the conditions for the exposure to light were changed. A solution of methyl orange and a suspension of zinc oxide were prepared in 5 mM sodium borate buffer (pH 9.2) and were mixed to give a final concentration of 40 μM and the equivalent of 6 mM, respectively. Test compounds were added at a final concentration of 100 μM, and each mixture was exposed to a 100-watt light from a distance of 20 cm for 2 h under gentle shaking. Blank mixtures pre-

* To whom correspondence should be addressed. e-mail: miwaichi@ccmfs.meijo-u.ac.jp

pared by the same procedure were placed in the dark for 2 h. Then each mixture was centrifuged at 1500×*g* for 5 min to remove the suspended zinc oxide. Photo-oxidation of the methyl orange by hydroxyl radicals, which were generated by photolysis of zinc oxide, was assessed by measuring the difference in the absorbance of methyl orange at 465 nm between the test solution and the blank solution.

Scavenging of Hydroxyl Radicals Produced by a Mixture of Fe^{3+}, EDTA, Ascorbate, and H_2O_2 Hydroxyl radical scavenging was measured by assessing the competition between deoxyribose and the test compounds for hydroxyl radicals generated from the Fe^{3+}/EDTA/ascorbate/H_2O_2 system. The hydroxyl radicals attack deoxyribose, resulting in the formation of thiobarbituric acid-reactive substances. The hydroxyl radical was generated by incubation of 20 mM sodium phosphate buffer (pH 7.4) containing 2.8 mM deoxyribose, 100 μM $FeCl_3$, 104 μM EDTA dipotassium salt, 100 μM ascorbic acid, and 1 mM H_2O_2, for 60 min at 37 °C, as described previously.[24] The incubation was performed in the presence and absence of the test compound. Solutions of $FeCl_3$ and ascorbic acid were made up immediately before use in deaerated water. An aliquot (100 μl) of each sample was mixed with 250 μl of water and 650 μl of 0.67% thiobarbituric acid, and the mixture was incubated at 95 °C for 30 min. After the mixture had been cooled in water, its absorbance was measured at 535 nm against a blank containing all of the reagents except deoxyribose.

Scavenging of Superoxide Radicals The superoxide radical was generated in a non-enzymatic system using phenazine methosulfate and NADH, and assayed by the reduction of nitro blue tetrazolium, according to the method of Robak and Gryglewski[25] except that the concentrations of phenazine methosulfate and NADH were doubled. The reaction mixture contained 20 μM phenazine methosulfate, 160 μM NADH, and 25 μM nitro blue tetrazolium in 100 mM sodium phosphate buffer (pH 7.4), with or without test compound. After a 5-min incubation of the above mixture at room temperature, the absorbance at 560 nm was read against blank samples that contained no phenazine methosulfate.

Scavenging of Hydrogen Peroxide Hydrogen peroxide-scavenging activity was assayed as described previously.[26] The reaction mixture (0.98 ml) contained phenol red (200 μM), H_2O_2 (10 μM), and test compound. After a 5-min incubation at room temperature, 10 μl of 10 units/ml horseradish peroxidase was added to the reaction mixture, which was then incubated for another 5 min to oxidize the phenol red by the remaining hydrogen peroxide. The reaction was terminated by adding 10 μl of 1 M NaOH. The purplish color of the oxidized phenol red was measured at 610 nm against blank samples containing no horseradish peroxidase.

Absorption Spectra Spectra of $CuSO_4$ (50 μM), chitobiose (100 μM), chitotriose (100 μM), $CuSO_4$+chitobiose, and $CuSO_4$+chitotriose in 100 mM sodium phosphate buffer (pH 7.4) were determined at room temperature.

Statistics Results were expressed as the mean±S.D. Statistical analysis was performed by using the unpaired Student's *t*-test. Values less than $p<0.05$ were accepted as significant.

RESULTS AND DISCUSSION

There is increasing evidence showing the involvement of oxidative stress in various diseases (*e.g.*, diabetes, atherosclerosis, cancer) and in aging. Consequently, the role of antioxidants in biological systems has received much attention. Extensive efforts to find antioxidant compounds usable for preventive and therapeutic purposes have been carried out by many workers.[27] In this study, we investigated the antioxidant activities of chitooligosaccharides, especially chitobiose and chitotriose, originating from natural sources. Because there is no simple universal method by which antioxidant activities can be measured accurately and quantitatively, we assessed the antioxidant activities of the test compounds in 5 different model systems.

First, we measured the inhibitory effects of chitooligosaccharides on hydroxylation of benzoate by H_2O_2 and compared them with those of *N*-acetylchitooligosaccharides (di-*N*-acetylchitobiose and tri-*N*-acetylchitotriose), monoasccharides (glucosamine, glucose, and fructose), sugar alcohol (sorbitol), disaccharide (maltose), aminoguanidine, pyridoxamine, and Trolox (Fig. 1). The last 3 compounds were used as reference compounds: both aminoguanidine[28,29] and pyridoxamine,[30,31] known to be beneficial in preventing diabetic complications in animals, have been reported to have antioxidant activity. The 4 chitooligosaccharides studied were all inhibitory against benzoate hydroxylation induced by H_2O_2, with chitobiose having a markedly high potency; whereas the other sugars studied did not show the inhibitory activity. The inhibitory activities of chitobiose, chitotriose, aminoguanidine, pyridoxamine, and Trolox were all dose dependent, with IC_{50} values of 18, 80, 85, 10, and 95 μM, respectively. The presence of more than 2 free amino groups in chitooligosaccharides may be mandatory for the antioxidant activity in this assay system, because glucosamine and *N*-acetylchitooligosaccharides were not effective at all.

The inhibitory activities of chitooligosaccharides and reference compounds against benzoate hydroxylation by H_2O_2 in the presence of various concentrations of $CuSO_4$ are

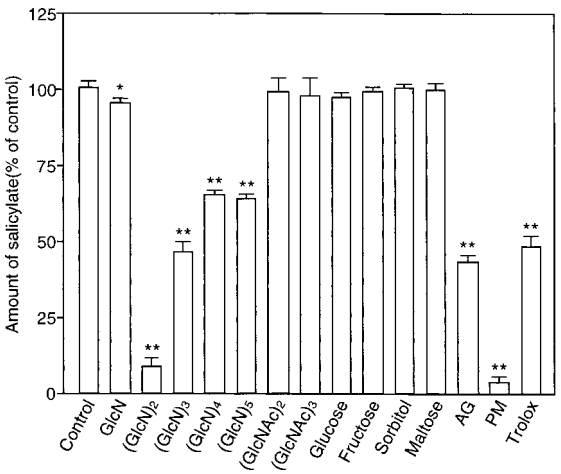

Fig. 1. Effects of Chitooligosaccharides and Other Compounds on the Hydroxylation of Benzoate by H_2O_2 in the Presence of 0.1 μM $CuSO_4$

The concentration of each compound tested was 100 μM. Values are means±S.D. of 3 independent determinations. GlcN, glucosamine; $(GlcN)_2$, chitobiose; $(GlcN)_3$, chitotriose; $(GlcN)_4$, chitotetraose; $(GlcN)_5$, chitopentaose; $(GlcNAc)_2$, di-*N*-acetylchitobiose; $(GlcNAc)_3$, tri-*N*-acetylchitotriose; AG, aminoguanidine; PM, pyridoxamine. $*p<0.05$, $**p<0.001$ as compared with the control.

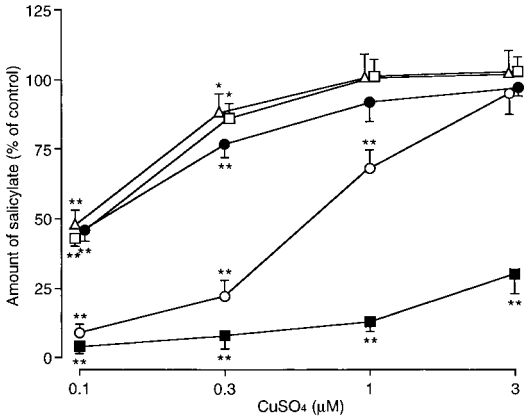

Fig. 2. Effects of Chitooligosaccharides and Other Compounds on the Hydroxylation of Benzoate by H_2O_2 in the Presence of Various Concentrations of $CuSO_4$

The concentration of the test compounds was 100 μM. Relative peak areas of salicylate in controls at 0.1, 0.3, 1, and 3 μM $CuSO_4$ were 1.0, 1.5, 1.8, and 2.1, respectively. ○, chitobiose; ●, chitotriose; □, aminoguanidine; ■, pyridoxamine; △, Trolox. Values are means±S.D. of 4 independent determinations. $*p<0.05$, $**p<0.001$ as compared with the respective control.

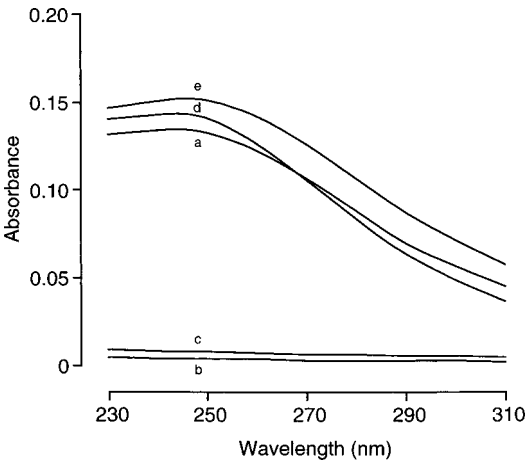

Fig. 3. Absorption Spectra of Cu^{2+}, Chitooligosaccharides, and Their Mixtures in 100 mM Sodium Phosphate Buffer (pH 7.4)

a, 50 μM $CuSO_4$; b, 100 μM chitobiose; c, 100 μM chitotriose; d, 50 μM $CuSO_4$+100 μM chitobiose; e, 50 μM $CuSO_4$+100 μM chitotriose.

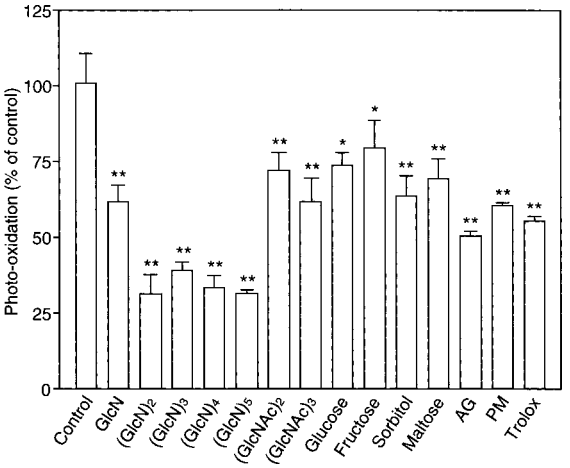

Fig. 4. Effects of Chitooligosaccharides and Other Compounds on the Photo-Oxidation of Methyl Orange

The concentration of each of the compounds tested was 100 μM. Values are means±S.D. of 6 independent determinations. See Fig. 1 for abbreviations. $*p<0.05$, $**p<0.001$ as compared with the control.

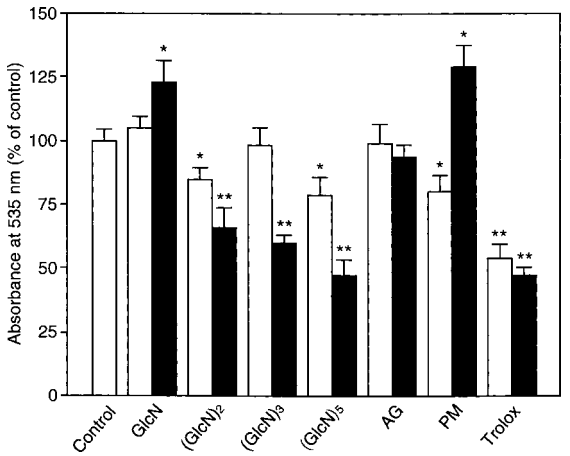

Fig. 5. Effects of Chitobiose, Chitotriose, and Other Compounds on Deoxyribose Degradation by Hydroxyl Radicals Produced by a Mixture of Fe^{3+}, EDTA, Ascorbate, and H_2O_2

The absorbance of the control was 0.581±0.028. Open column, 100 μM test compound; closed column, 1 mM test compound. Values are means±S.D. of 4 independent determinations. See Fig. 1 for abbreviations. $*p<0.05$, $**p<0.001$ as compared with the control.

shown in Fig. 2. All of the compounds tested were less inhibitory as the $CuSO_4$ concentration was raised, suggesting that the chelation of these compounds with Cu^{2+} would be involved in the antioxidant activity in this assay system. This finding is consistent with the fact that H_2O_2-induced benzoate hydroxylation did not take place when the reaction mixture was treated with Chelex-100 and then incubated in the absence of $CuSO_4$. Our view is also supported by the observation that aminoguanidine and pyridoxamine inhibited Cu^{2+}-catalyzed autoxidation of ascorbic acid by chelating with Cu^{2+}.[30] In order to obtain direct evidence for the complex formation of chitooligosaccharides with Cu^{2+}, we measured the absorbance of Cu^{2+}, chitooligosaccharides, and their mixtures. The absorption spectrum of Cu^{2+} in the ultraviolet region was appreciably changed by the presence of chitobiose or chitotriose (Fig. 3), whereas maltose did not affect it (data not shown). This indicates that chitooligosaccharides interact with Cu^{2+} to form chelates.

The scavenging activities of chitooligosaccharides and other compounds towards hydroxyl radicals produced by photolysis of zinc oxide are shown in Fig. 4. All of the compounds elicited the hydroxyl radical-scavenging activity, and the 4 chitooligosaccharides were the most effective among them. The scavenging activities of chitobiose, chitotriose, aminoguanidine, pyridoxamine, and Trolox were all dose dependent, with IC_{50} values of 30, 55, 100, 125, and 118 μM, respectively. It is conceivable also in this assay system that the presence of more than 2 free amino groups in chitooligosaccharides is needed for the antioxidant activity.

The 3 chitooligosaccharides studied dose-dependently scavenged hydroxyl radicals produced by a mixture of Fe^{3+}, EDTA, ascorbate, and H_2O_2 when tested at 0.1 and 1 mM (Fig. 5). The ability of chitooligosaccharides to scavenge hydroxyl radicals was thus demonstrated in both experimental systems, as shown in Figs. 4 and 5. The reason why glucosamine and pyridoxamine at 1 mM stimulated the degradation of deoxyribose by hydroxyl radicals (Fig. 5) remains to

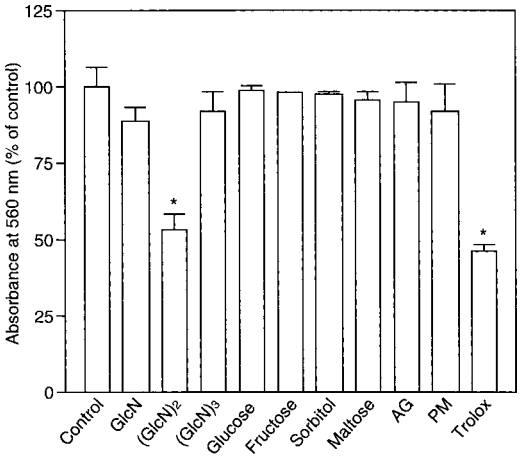

Fig. 6. Effects of Chitobiose, Chitotriose, and Other Compounds on the Reduction of Nitro Blue Tetrazolium by Superoxide Radicals Generated by Phenazine Methosulphate and NADH

The absorbance of the control was 0.452±0.024. The concentration of each compound tested was 1 mM. Values are means±S.D. of 4 independent determinations. See Fig. 1 for abbreviations. $*p<0.001$ as compared with the control.

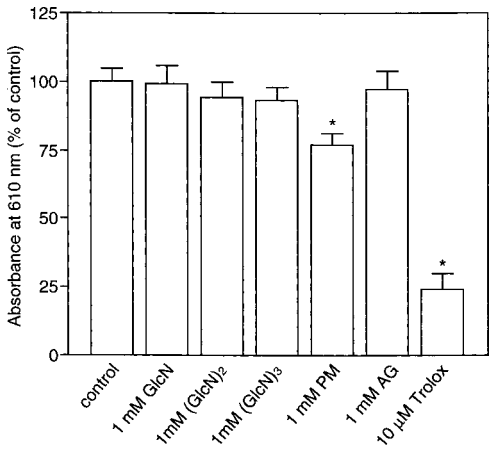

Fig. 7. Scavenging Activities of Chitobiose, Chitotriose, and Other Compounds towards Hydrogen Peroxide

The absorbance at 610 nm induced by the peroxidase-mediated oxidation of phenol red in the control was 0.405±0.022. Values are means±S.D. of 4 independent determinations. See Fig. 1 for abbreviations. $*p<0.001$ as compared with the control.

be answered.

Only chitobiose and Trolox of the 10 compounds tested exhibited superoxide radical-scavenging activity (Fig. 6). Other investigators[32] also reported that Trolox had the ability to scavenge superoxide radicals in the same assay system as ours. The finding that only the dimer, but not the monomer and the trimer, of glucosamine had the scavenging activity towards superoxide radicals may suggest the importance of the conformation of chitobiose in the activity.

Neither chitobiose nor chitotriose at 1 mM showed the hydrogen peroxide scavenging activity, whereas Trolox at 10 μM effectively scavenged hydrogen peroxide (Fig. 7). The high potency of Trolox in scavenging hydrogen peroxide was shown also in a previous paper.[32]

Antioxidants, in general, can influence the peroxidation process through either a simple or complex mechanisms including free-radical scavenging, divalent-metal chelation, and so on. The present study implicates the abilities of chitooligosaccharides to scavenge hydroxyl radicals and superoxide radicals, but not hydrogen peroxide, and to chelate divalent metals in their antioxidant activities. The reactions involved in scavenging hydroxyl radicals and superoxide radicals, however, remain to be solved.

Chitobiose and chitotriose, but not chitotetraose and chitopentaose, were absorbed into the rat body in amounts that reached plasma concentrations of about 20 and 10 μM, respectively, when given once by gavage at a dose of 100 mg/kg (A.-S. C., T.T., T.N., and I.M., unpublished observation). Taken together with this observation, our results suggest that chitobiose and chitotriose would act as effective antioxidants *in vivo* when orally ingested, with a greater potency for the former than for the latter.

Acknowledgements This work was supported by grant-in-aids (to I.M.) for the High-Tech Research Center Project and for the Scientific Frontier Research Project from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1) Hirano S., Nagao N., *Agric. Biol. Chem.*, **53**, 3065—3066 (1989).
2) Tsai G. J., Su W. H., *J. Food Prod.*, **62**, 239—243 (1999).
3) Miura T., Usami M., Tsuura Y., Ishida H., Seino Y., *Biol. Pharm. Bull.*, **18**, 1623—1625 (1995).
4) Kondo Y., Nakatani A., Hayashi K., Ito M., *Biol. Pharm. Bull.*, **23**, 1458—1464 (2000).
5) Hayashi K., Ito M., *Biol. Pharm. Bull.*, **25**, 188—192 (2002).
6) Okamoto Y., Shibazaki K., Minami S., Matsuhashi A., Tanioka S., Shigemasa Y., *J. Vet. Med. Sci.*, **57**, 851—854 (1995).
7) Stone C. A., Wright H., Clarke T., Powell R., Devaraj V. S., *Br. J. Plast. Surg.*, **53**, 601—606 (2000).
8) Gallaher C. M., Munion J., Hesslink R., Jr., Wise J., Gallaher D. D., *J. Nutr.*, **130**, 2753—2759 (2000).
9) Tai T.-S., Sheu W. H.-H., Lee W.-J., Yao H.-T., Chang M.-T., *Diabetes Care*, **23**, 1703—1704 (2000).
10) Ylitalo R., Lehtinen S., Wuolijoki E., Ylitalo P., Lehtimäki T., *Arzneim.-Forsch./Drug Res.*, **52**, 1—7 (2002).
11) Singla A. K., Chawla M., *J. Pharm. Pharmacol.*, **53**, 1047—1067 (2001).
12) Trautwein E. A., Jürgensen U., Erbersdobler H. F., *Nutr. Res.*, **17**, 1053—1065 (1997).
13) Deuchi K., Kanauchi O., Shizukuishi M., Kobayashi E., *Biosci. Biotech. Biochem.*, **59**, 1211—1216 (1995).
14) Kim S.-K., Park P.-J., Yang H.-P., Han S.-S., *Arzneim.-Forsch./Drug Res.*, **51**, 769—774 (2001).
15) Choi B.-K., Kim K.-Y., Yoo Y.-J., Oh S.-J., Choi J.-H., Kim C.-Y., *Int. J. Antimicrobi. Agents*, **18**, 553—557 (2001).
16) Kendra D. F., Hadwiger L. A., *Exp. Mycol.*, **8**, 276—281 (1984).
17) Xue C., Yu G., Hirata T., Terao J., Lin H., *Biosci. Biotechnol. Biochem.*, **62**, 206—209 (1998).
18) Nam K.-S., Choi Y.-R., Shon Y.-H., *Biotechnol. Lett.*, **23**, 971—975 (2001).
19) Pae H.-O., Seo W.-G., Kim N.-Y., Oh G.-S., Kim G.-E., Kim Y.-H., Kwak H.-J., Yun Y.-G., Jun C.-D., Chung H.-T., *Leuk. Res.*, **25**, 339—346 (2001).
20) Suzuki K., Mikami T., Okawa Y., Tokoro A., Suzuki S., Suzuki M., *Carbohydr. Res.*, **151**, 403—408 (1986).
21) Tokoro A., Tatewaki N., Suzuki K., Mikami T., Suzuki S., Suzuki M., *Chem. Pharm. Bull.*, **36**, 784—790 (1988).
22) Giardino I., Fard A. K., Hotchell D. L., Brownlee M., *Diabetes*, **47**, 1114—1120 (1998).
23) Russell J., Ness J., Chopra M., McMurray J., Smith W. E., *J. Pharmaceut. Biomed. Anal.*, **12**, 863—866 (1994).
24) Halliwell B., Gutteridge J. M. C., Aruoma O. I., *Anal. Biochem.*, **165**, 215—219 (1987).
25) Robak J., Gryglewski R. J., *Biochem. Pharmacol.*, **37**, 837—841 (1988).

26) Wei H., Wei L., Frenkel K., Bowen R., Barnes S., *Nutr. Cancer*, **20**, 1—12 (1993).
27) Niki E., *Free Rad. Res.*, **33**, 693—704 (2000).
28) Picard S., Parthasarathy S., Fruebis J., Witztum J., *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 6876—6880 (1992).
29) Kedziora-Kornatowska K., Luciak M., *Biochem. Mol. Biol. Int.*, **46**, 577—583 (1998).
30) Price D. L., Rhett P. M., Thorpe S. R., Baynes J. W., *J. Biol. Chem.*, **276**, 48967—48972 (2001).
31) Jain S. K., Lim G., *Free Radic. Biol. Med.*, **30**, 232—237 (2001).
32) Yang M.-L., Huang T.-S., Lee Y., Lu F.-J., *Free Rad. Res.*, **36**, 685—693 (2002).