Effects of Nonylphenol and Triclosan on Production of Plasma Vitellogenin and Testosterone in Male South African Clawed Frogs (*Xenopus laevis*)

Naomi MATSUMURA,[a] Hiroshi ISHIBASHI,[a] Masashi HIRANO,[a] Yukiko NAGAO,[a] Naoko WATANABE,[a] Hideki SHIRATSUCHI,[a] Toshinori KAI,[a] Tetsuji NISHIMURA,[b] Akihiko KASHIWAGI,[c] and Koji ARIZONO*,[a]

[a] *Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto; 3–1–100 Tsukide, Kumamoto 862–8502, Japan:* [b] *National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan: and* [c] *Institute for Amphibian Biology, Graduate School of Science, Hiroshima University; 1–3–1 Kagamiyama, Higashihiroshima 739–8526, Japan.* Received March 23, 2005; accepted June 6, 2005; published online June 14, 2005

We investigated the effects of nonylphenol (NP) and triclosan (TCS) on production of vitellogenin (Vg), testosterone (T), and hepatic cytochrome P450 1A and 2B activities in male South African clawed frogs (*Xenopus laevis*). In a 14-d waterborne exposure test, no significant differences in the level of plasma Vg synthesis in male frogs were observed among the control, 10, 50, and 100 μg/l NP and 20, 100, and 200 μg/l TCS treatment groups. Intraperitoneal injection of male frogs with 2, 20, and 200 μg/g body weight NP resulted in no significant differences in plasma Vg levels among the control and all treatment groups. However, the levels of plasma Vg in all TCS treatment groups (intraperitoneal injection of 4, 40, and 400 μg/g body weight) were lower than that in the solvent control group, and male frogs injected with high doses of NP or TCS had lower T levels than the control group. No significant differences in hepatic cytochrome P450 1A and 2B activities were observed among the all treatment groups. Male frogs injected with 20 μg/g body weight of estradiol-17β had significantly higher plasma Vg levels than the control group. These results suggest that profiles of plasma Vg and T production in male *Xenopus laevis* could be useful biomarkers for detecting hormonally active agents.

Key words *Xenopus laevis*; vitellogenin; nonylphenol; triclosan

Recently, a number of studies have been performed worldwide to examine endocrine-disrupting chemicals (EDCs) and their interactions with the development and function of various systems in animals and humans.[1—3] Among these EDCs, alkylphenol polyethoxylate non-ionic surfactants are used in the manufacture of cleaning agents, cosmetics and food products, as well as in plastic polymerization processes. Nonylphenol ethoxylates have predominantly been used, amounting to about 80% of the production of alkylphenol surfactants. In a recent study, nonylphenol (NP) had significant effects on the reproductive potential of medaka (*Oryzias latipes*) at concentrations as low as 17.7 μg/l,[4] and 50% of the male fish in the 50 μg/l treatment and 86% of the males in the 100 μg/l treatment developed testis-ova, an intersex condition characterized by both testicular and ovarian tissue in the gonad.[5] On the other hand, triclosan (TCS, 2,4,4′-trichloro-2′-hydroxydiphenyl ether) is widely used as an antibacterial agent in liquid toothpaste, soap, shampoo, and cosmetics, and is frequency found in wastewater effluent. Water samples collected near the outfall of a wastewater treatment plant in Rhode Island, U.S.A., showed 10—20 μg/l of TCS in the effluent and 80—100 μg/g of TCS in the sediment.[6,7] Our previous study suggests that TCS has high toxicity on the early life stages of medaka, and that the metabolite of TCS may be a weak estrogenic compound in male medaka but with no adverse effect on reproductive success (such as fecundity and fertility) and offspring.[8] Moreover, Hanioka *et al.*[9,10] reported that 7-benzyloxyresorufin *O*-debenzylase (BROD) and 7-pentoxyresorufin *O*-depentylase (PROD) activities, which are associated with CYP2B1 activity, were remarkably induced by all doses of TCS in rats. Their results suggested that TCS induces the P450 isoforms of the CYP2B subfamily in the rat liver, and that the induced P450 isozymes were closely related to the toxicity of TCS or its chlorinated derivatives. However, there is no information about the effects of these chemicals on the hormonally responses of amphibian.[11]

The South African clawed frog (*Xenopus laevis*) is sensitive to environmental chemicals as it spends all the life stages (egg, larva, and adulthood) in water. Hayes *et al.*[12] examined the effects of atrazine on sexual development in *X. laevis*, and reported that atrazine ($>$ or $=0.1$ μg/l) induced hermaphroditism and demasculinized the larynx of exposed males ($>$ or $=1.0$ μg/l), and male *X. laevis* suffered a 10-fold decrease in testosterone levels when exposed to 25 μg/l atrazine. Therefore, in the present study, we used *X. laevis* as a test organism, and investigated the effects of NP and TCS on production of plasma vitellogenin (Vg, egg yolk protein precursor), steroid hormone testosterone (T) synthesis, and hepatic CYP1A and CYP2B, as measured by the ethoxyresorufin *O*-deethylase (EROD) or PROD activities in male *X. laevis*. To our knowledge, this is first study on the effect of TCS in male *X. laevis*.

MATERIALS AND METHODS

Test Chemicals TCS (>98.0% purity) was obtained from Wako Pure Chemical Industries, Ltd., Tokyo, Japan. NP (technical grade; mixture of ring and chain isomers) was obtained from Aldrich Chemical Company Inc., Tokyo, Japan. These chemicals were dissolved in dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries) and propylene glycol (Wako Pure Chemical Industries) to prepare test solutions.

Test Organism Male *X. laevis* (body weight; approximately 50—60 g) were purchased from Kato S Science,

* To whom correspondence should be addressed. e-mail: arizono@pu-kumamoto.ac.jp

Chiba, Japan, and have been maintained in glass tanks in our laboratory under 16 h light–8 h dark photoperiod at 24±1 °C. The frogs were fed a commercial diet (Kato S Science) once daily during the acclimatization period for 7 d.

Exposure Design Frogs were exposed to the chemicals either *via* water or intraperitoneal injection. In the waterborne exposure, each group of frogs (six frogs per treatment) was exposed to the nominal concentrations of 10, 50, and 100 μg/l NP, and 20, 100, and 200 μg/l TCS dissolved in dechlorinated tap water for 14 d. The control frogs were exposed to the solvent carrier only (DMSO 0.1 ml/l), and the positive control frogs were exposed to the nominal concentration of 1 μg/l of estradiol-17β (E2). The exposure periods of these chemicals were selected on the basis of the results of a previous study.[13] During the experimental period, the exposure water in the tanks was changed every 24 h. Blood samples were then taken at the end of the exposure period.

In the intraperitoneal exposure, each group of frogs (six frogs per treatment) was injected with 2, 20, and 200 μg/g body weight NP, and 4, 40, and 400 μg/g body weight TCS dissolved in propylene glycol. The control frogs were injected with the solvent carrier only (propylene glycol), and the positive control frogs were injected with 20 μg/g body weight of E2. After the injection, frogs were transferred to glass tanks containing dechlorinated tap water, and maintained for 7 d. Blood samples were then taken at the end of the exposure period.

In both exposure systems, each group of frogs was kept in a 30-l glass tank maintained under 16 h light–8 h dark photoperiod at 24±1 °C, and fed 0.3% body weight of a commercial diet every day during the experimental period.

Preparation of Blood and Microsome Samples Frogs were weighed and blood samples were taken from the caudal vasculature with heparinized syringe and needle. Blood samples were transferred into a centrifuge tube, and centrifuged at 6000×g for 10 min. The plasma was then stored at −80 °C until assayed. All preparative procedures were carried out at 4 °C.

The liver was homogenized with 4 volumes of 0.25 M sucrose in a Potter-Elvehjem homogenizer with a Teflon pestle. Preparation of the 105000×g soluble fraction and microsomes was carried out by the procedures previously described by Arizono *et al.*[14] and Ariyoshi *et al.*,[15] respectively. Microsomal protein content was determined according to Lowry *et al.*[16] using bovine serum albumin as a standard. The microsomal samples were stored at −80 °C until use. All preparative procedures were carried out at 4 °C.

Measurement of Plasma Vg Plasma Vg levels in male frog were measured using enzyme-linked immunosorbent assay (ELISA) in a Vg assay kit (Japan EnviroChemicals, Osaka, Japan) specifically for frog developed by Mitsui *et al.*[13] The measurement of plasma Vg was performed according to the manufacture's procedure. Purified frog Vg (1—1000 ng/ml) was used as a standard, and Vg in diluted samples was measured in duplicate. The assays were performed at room temperature. Concentrations of Vg in plasma samples were calculated from the linear part of the log-transformed frog Vg standard curve. The detection limit of Vg in the present study was 1 ng/ml.

Measurement of Plasma T Plasma T levels were measured by an ELISA as described by Ishibashi *et al.*[17] T was extracted from blood plasma three times using ten volumes of diethyl ether. The ether was evaporated under nitrogen, and the sample was reconstituted with assay buffer. Each sample was analyzed in duplicate for T corrected for extraction efficiencies of 90%. The minimum concentration detectable was 50 pg/ml for T. In this ELISA system, inter-assay and intra-assay coefficients of variation were less than 10% for plasma T.

Measurement of Hepatic EROD and PROD Activities EROD and PROD activities in frog liver microsomes were measured by dealkylation of ethoxyresorufin and pentoxyresorufin, respectively, and detection of the resulting resorufin by high-performance liquid chromatography (HPLC) with fluorescent detection as described by Tatarazako *et al.*[18]

Statistical Analysis All statistical analyses were performed using Stat View J 5.0 (SAS Institute Inc., Cary, North Carolina, U.S.A.). All experimental data were checked for assumptions of homogeneity of variance across treatments using a Bartlett test. When the assumptions were met, data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison tests.[19] When no homogeneity was observed in the data, nonparametric Kruskal–Wallis test was used, followed by a Mann–Whitney *U* test with Bonferroni's adjustment.[20] Differences were considered significant at $p<0.05$ or $p<0.01$.

RESULTS AND DISCUSSION

In this study, we investigated the effects of NP and TCS on production of Vg, T, and hepatic EROD or PROD activities in male *X. laevis*. In the waterborne exposure, there were no significant differences in body weight among the solvent control, NP- or TCS-treated groups (ranging from 51.7 to 62.8 g). In addition, the intraperitoneal injection, there were no significant differences in body weight among the solvent control and NP- or TCS-treated groups (ranging from 53.3 to 62.0 g); however, all the animals injected 200 μg/g body weight of NP died during the experimental period.

In the waterborne exposure, there were no significant differences in plasma Vg levels among the NP- or TCS-treated groups (Fig. 1). The 10 μg/l NP tested in this study was often detected in the aquatic environment, and 50 and/or 100 μg/l NP caused the induction of Vg in fish.[21—23] On the other hand, water samples collected near the outfall of a wastewater treatment plant in Rhode Island, U.S.A., showed 10—20 μg/l of TCS in the effluent.[6] These results indicate that NP or TCS did not have any estrogenic effects on adult male frog at the concentrations detected in the aquatic environment. Furthermore, high levels of E2-1 μg/l did not induce plasma Vg in male frog in the present study (Fig. 1), although it is known that endogenous estrogen such as E2 induced the plasma and/or hepatic Vg in fish.[24,25]

In the intraperitoneal injection, we could not demonstrate the induction of plasma Vg in male *X. laevis* at NP concentrations tested in this study as well as in result of water exposure (Fig. 2). Van Wyk *et al.*[11] reported that no induction of plasma Vg in male *X. laevis* treated with 100 μg NP/g/week. Therefore, NP may not have estrogenic effect in adult male *X. laevis*. Moreover, male frogs injected with high doses of NP had lower T levels than the control group, although no statistically significant difference was determined because a

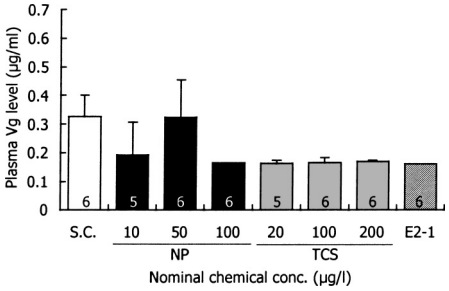

Fig. 1. Levels of Plasma Vitellogenin (Vg) Concentration of Male Frogs (*Xenopus laevis*) Treated with Nonylphenol (NP) and Triclosan (TCS) in Water Exposure for 14 d

The control frogs (S.C.) were exposed to the solvent carrier only (DMSO 0.1 ml/l), and the positive control frogs were exposed to the nominal concentration of 1 μg/l of estradiol-17β (E2). Data represent at the mean±S.D. The number in each bar represents the number of individuals.

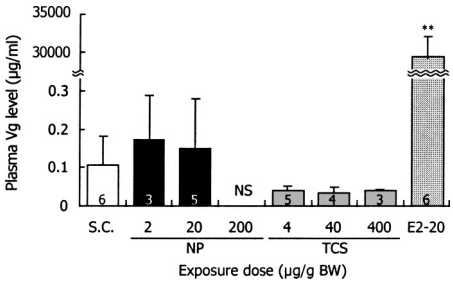

Fig. 2. Levels of Plasma Vitellogenin (Vg) Concentration of Male Frogs (*Xenopus laevis*) Treated with Nonylphenol (NP) and Triclosan (TCS) in Intraperitoneal Injection

The control frogs (S.C.) were injected with the solvent carrier only (propylene glycol), and the positive control frogs were injected with dose of 20 μg/g body weight of estradiol-17β (E2). NS; no samples. ** Significant difference compared to the control frogs ($p<0.01$). Data represent at the mean±S.D. The number in each bar represents the number of individuals.

large individual variation was observed (Fig. 3). Similar observations have been reported in *X. laevis* exposed to NP.[11] It might be that NP inhibited an aspect(s) of steroid release and/or synthesis common to T in male *X. leaevis*. However, Okoumassoun *et al*.[26] assessed the estrogenic activity of the organochlorine pesticides *o*,*p*′-DDT, dieldrin, aldrin, heptachlor, mirex and DDT in rainbow trout hepatocyte cultures using Vg. Heptachlor and mirex did not induce Vg. They suggested that the EC_{50} value for inhibition of estrogen receptor binding by heptachlor was cytotoxic for hepatocytes in culture, and this could in part explain the lack of Vg response observed with this compound at the concentrations tested. In this study, although there were no significant differences in body weight among all treatment groups, all frogs injected with 200 μg/g body weight of NP died during the experimental period. Thus, no Vg production in male frogs exposed to NP may cause lethal toxicity.

The levels of plasma Vg in all TCS treatment groups were lower than that in the solvent control group in the intraperitoneal injection, but no statistically significant difference was determined (Fig. 2). In addition, male frogs injected with high doses of TCS had lower T levels than the control group, but no statistically significant difference was determined (Fig. 3). Panter *et al*.[27] reported that while the pharmaceutical antiestrogen ZM 189,154 had no impact on somatic endpoints in juvenile fathead minnow (*Pimephales promelas*), this substance caused a reduction in Vg concentrations,

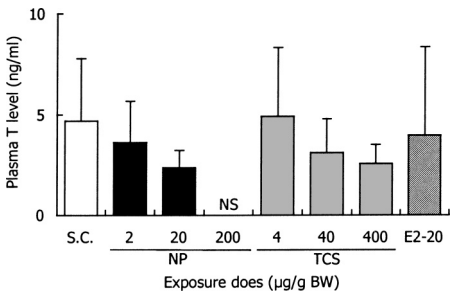

Fig. 3. Levels of Plasma Testosterone (T) Concentration of Male Frogs (*Xenopus laevis*) Treated with Nonylphenol (NP) and Triclosan (TCS) in Intraperitoneal Injection

The control frogs (S.C.) were injected with the solvent carrier only (propylene glycol), and the positive control frogs were injected with dose of 20 μg/g body weight of estradiol-17β (E2). NS; no samples. Data represent at the mean±S.D. The number of each treatment group is shown in Fig. 2.

which is consistent with its mode of action as an estrogen receptor antagonist. Recent studies have demonstrated changes in fin length and non-significant trends in sex ratio of medaka (*Oryzias latipes*) exposed to 1—100 μg/l TCS detected in aquatic environment, and have suggested that TCS has weak androgenic and antiestrogenic potency.[28] Our previous study also suggests that the metabolite of TCS may be a weak estrogenic compound in male medaka.[8] Therefore, the reduction of plasma Vg in this study may be due to the antiestrogenic effects of TCS in male *X. laevis*.

In the present study, male frogs injected with 20 μg/g body weight of E2 had significantly higher plasma Vg levels than the solvent control group (Fig. 2). Van Wyk *et al*.[11] also reported that treatment with 10 μg/g/week of E2 induced plasma Vg synthesis in male *X. laevis*. These results suggest that induction of plasma Vg in male *X. laevis* could be a suitable biomarker for the evaluation of estrogenic activity of EDCs.

Fujita *et al*.[29] reported molecular cloning and sequence analysis of cDNAs coding for 3-methylcholanthrene-inducible CYP1A isoform in *X. laevis* liver. In contrast, induction of hepatic CYP2B by phenobarbital (PB) has been reported in semi-aquatic frog (*Rana pipiens*).[30] Therefore, the determinations of EROD and PROD activities in frog are important markers of toxicological research.[30] Hanioka *et al*.[9,10] reported that BROD and PROD activities in rats, were induced by all doses of TCS, suggesting that TCS induced the hepatic P450 isoforms of the CYP2B subfamily. We also assessed the induction of EROD and PROD activities in hepatic microsomes from male frog exposed to TCS but found no difference between the EROD and PROD activities among the TCS treatment groups (Fig. 4). Although PB induction of CYP2B has not been clarified in *X. laevis*, it is unclear whether our results are due to physiological differences in the induction of hepatic CYP2B between *X. laevis* and *R. pipiens*. Further experiments in the laboratory are required to evaluate CYP2B induction in *X. laevis* exposed to various CYPs inducers.

Acknowledgments We should like to express our grateful thanks to Mr. Noriaki Santo and Dr. Osamu Tooi (Towa Kagaku Co., Ltd., Hiroshima, Japan) for technical advice. The authors would like to thank Dr. Charles Sagoe (Towa Kagaku Co., Ltd., Hiroshima, Japan) for critical reading of

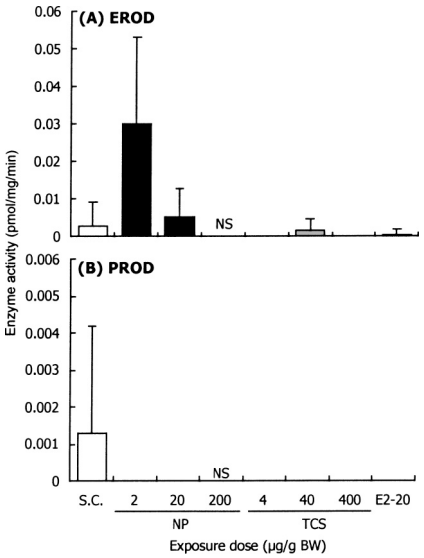

Fig. 4. EROD and PROD Activities in Microsome of Male Frogs (*Xenopus laevis*) Treated with Nonylphenol (NP) and Triclosan (TCS) in Intraperitoneal Injection

The control frogs (S.C.) were injected with the solvent carrier only (propylene glycol), and the positive control frogs were injected with dose of 20 μg/g body weight of estradiol-17β (E2). NS; no samples. Data represent at the mean±S.D. The number of each treatment group is shown in Fig. 2.

manuscript. We thank Towa Kagaku Co., Ltd. (Hiroshima, Japan) and Japan EnviroChemicals (Osaka, Japan) for providing the *X. laevis* Vg ELISA kit. This study was supported in part by funds from the Environment Agency, Japan.

REFERENCES

1) Colborn T., Dumanoshi D., Myers J. P., "Our Stolen Future," Dutton, NY, 1996.
2) Harries J. E., Sheahan D. A., Jobling S., Matthiessen P., Neall P., Routledge E., Rycroft R., Sumpter J. P., Tylor T., *Environ. Toxicol. Chem.*, **15**, 1993—2002 (1996).
3) Semenza J. C., Tolbert P. E., Rubin C. H., Guillette L. J., Jr., Jackson R. J., *Environ. Health Perspect*., **105**, 1030—1032 (1997).
4) Yokota H., Seki M., Maeda M., Oshima Y., Tadokoro H., Honjo T., Kobayashi K., *Environ. Toxicol. Chem.*, **20**, 2552—2560 (2001).
5) Michelle A. G., Chris D. M., *Environ. Toxicol. Chem.*, **16**, 1082—1086 (1997).
6) McAvoy D. C., Schatowitz B., Jacob M., Hauk A., Eckhoff W. S., *Environ. Toxicol. Chem.*, **21**, 1323—1329 (2002).
7) Singer H., Muller S., Tixier C., Pillonel L., *Environ. Sci. Technol*., **36**, 4998—5004 (2002).
8) Ishibashi H., Matsumura N., Hirano M., Matsuoka M., Shiratsuchi H., Ishibashi Y., Takao Y., Arizono K., *Aquat. Toxicol*., **67**, 167—179 (2004).
9) Hanioka N., Omae E., Nishimura T., Jinno H., Onodera S., Yoda R., Ando M., *Chemosphere*, **33**, 265—276 (1996).
10) Hanioka N., Jinno H., Nishimura T., Ando M., *Chemosphere*, **34**, 719—730 (1997).
11) Van Wyk J. H., Pool E. J., Leslie A. J., *Arch. Environ. Contam. Toxicol.*, **44**, 247—256 (2003).
12) Hayes T. B., Collins A., Lee M., Mendoza M., Noriega N., Stuart A. A., Vonk A., *Proc. Natl. Acad. Sci. U.S.A.*, **99**, 5476—5480 (2002).
13) Mitsui N., Tooi O., Kawahara A., *Comp. Biochem. Physiol. C Toxicol. Pharmacol.*, **135**, 305—313 (2003).
14) Arizono K., Ito T., Yamaguchi M., Ariyoshi T., *Eisei Kagaku*, **28**, 94—98 (1982).
15) Ariyoshi T., Takabatake E., Remmer H., *Life Sci*., **9**, 361—369 (1970).
16) Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
17) Ishibashi H., Tachibana K., Tsuchimoto M., Soyano K., Tatarazako N., Matsumura N., Tomiyasu Y., Tominaga N., Arizono K., *Comp. Med.*, **54**, 54—62 (2004).
18) Tatarazako N., Takigami H., Koshio M., Kawabe K., Hayakawa Y., Arizono K., Morita M., *Environ. Sci.*, **9**, 451—462 (2002).
19) Dunnett C. W., *J. Am. Stat. Assoc.*, **50**, 1096—1121 (1995).
20) Glantz S. A., "Primer of Biostatistics," 3rd ed., McGraw-Hill, New York, 1992.
21) Villeneuve D. L., Villalobos S. A., Keith T. L., Snyder E. M., Fitzgerald S. D., Giesy J. P., *Chemosphere*, **47**, 15—28 (2002).
22) Seki M., Yokota H., Maeda M., Tadokoro H., Kobayashi K., *Environ. Toxicol. Chem.*, **22**, 1507—1516 (2003).
23) Hill R. L., Jr., Janz D. M., *Aquat. Toxicol.*, **63**, 417—429 (2003).
24) Kang I. J., Yokota H., Oshima Y., Tsuruda Y., Yamaguchi T., Maeda M., Imada N., Tadokoro H., Honjo T., *Chemosphere*, **47**, 71—80 (2002).
25) Rose J., Holbech H., Lindholst C., Norum U., Povlsen A., Korsgaard B., Bjerregaard P., *Comp. Biochem. Physiol. C Toxicol. Pharmacol.*, **131**, 531—539 (2002).
26) Okoumassoun L. E., Averill-Bates D., Gagne F., Marion M., Denizeau F., *Toxicology*, **178**, 193—207 (2002).
27) Panter G. H., Hutchinson T. H., Lange R., Lye C. M., Sumpter J. P., Zerulla M., Tyler C. R., *Environ. Toxicol. Chem.*, **21**, 319—326 (2002).
28) Foran C. M., Bennett E. R., Benson W. H., *Mar. Environ. Res*., **50**, 153—156 (2000).
29) Fujita Y., Ohi H., Murayama N., Saguchi K., Higuchi S., *Arch. Biochem. Biophys.*, **371**, 24—28 (1999).
30) Khan M. A. Q., Qadri S. Y., Tomar S., Fish D., Gururajan L., Poria M. S., *Biochem. Biophys. Res. Commun.*, **244**, 737—744 (1998).