*Biochemistry (Moscow), Vol. 66, No. 3, 2001, pp. 282-285. Translated from Biokhimiya, Vol. 66, No. 3, 2001, pp. 349-353.*


# Luminol-Dependent Chemiluminescence Analysis of Chitooligosaccharide-Induced Rapid Production of Hydrogen Peroxide by Intact Wheat Seedlings

**R. M. Khairullin* and I. E. Akhmetova**

*Institute of Biochemistry and Genetics, Ufa Scientific Center, Russian Academy of Sciences, pr. Oktyabrya 69, Ufa, 450054 Russia; fax: (3472) 35-6100; E-mail: phyto@anrb.ru*

Received April 20, 2000
Revision received November 16, 2000

**Abstract**—The feasibility of a luminol-dependent chemiluminescence analysis of hydrogen peroxide production by intact wheat seedlings using a KhL-003 chemiluminometer was determined. It was shown that the minimal $H_2O_2$ concentration that can be detected in a 0.5-ml sample with this instrument is 0.125 μM. Analysis of biological activity of a mixture of chitooligosaccharides with molecular masses from 5 to 10 kD and acetylation degree of 65% demonstrated that, at a concentration of 1 μg/ml, they induce rapid overproduction of $H_2O_2$ in roots of 3-day-old wheat seedlings.

*Key words*: hydrogen peroxide, chemiluminescence analysis, wheat seedlings, chitooligosaccharides

The production of hydrogen peroxide in plants infected by pathogens or treated with elicitors, substances evoking defense reactions, has been the focus of intensive research [1-3]. This interest is due to two factors: first, extremely rapid production of $H_2O_2$ (2-5 min) makes it possible to consider this process as one of the primary reactions of cells to external factors, and second, a signaling role of hydrogen peroxide in living organisms is discussed in the literature [4, 5]. Note that in many works dealing with this problem cell cultures or plant organs are used.

Among the elicitors inducing rapid $H_2O_2$ production in plant cells, the role of glycoproteins, cell wall constituents of many phytopathogenic fungi, has been most studied [2, 6]. Although cell walls of fungi are rich in chitin and chitosan, their role in $H_2O_2$ formation in plants is poorly understood. It was shown that these elicitors activate various plant enzymes such as chitinase [7], peroxidase [8], phenylalanine-ammonium lyase [9], etc.

Problems associated with plant cultivation *in vitro* and the lack of simple procedures for determination of hydrogen peroxide in plants impede progress in this field. We explored the possibility of measuring $H_2O_2$ production in intact wheat plants using a KhL-003 chemiluminometer. We also studied the ability of a mixture of chitin–chitosan oligomers (chitooligosaccharides, CHOS) to induce $H_2O_2$ production in wheat seedlings.

* To whom correspondence should be addressed.

## MATERIALS AND METHODS

Seeds of common wheat *Triticum aestivum* L. (cv. Moskovskaya 35) were sterilized in 80% ethanol for 5 min, washed in distilled water, and germinated on sterile filter paper for three days at room temperature in the dark. The seedlings were separated from endosperm, washed in distilled water, and transferred for 6 h to potassium phosphate buffer (0.01 M $KH_2PO_4/K_2HPO_4$ and 1 mM $CaCl_2$ dissolved in twice distilled water and adjusted to pH 6.5) (analytical grade, Reakhim, Russia) containing chloramphenicol (Serva, Germany) (10 mg/liter). The growth medium was substituted for fresh medium without chloramphenicol 30 min before the experimentation.

Determination of relative $H_2O_2$ concentrations in the medium was based on chemiluminescence emission of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) oxidized in the presence of hydrogen peroxide and peroxidase. Chemiluminescence was measured on a KhL-003 instrument developed in the Joint Laboratory of the Ufa Aviation Technical University and Bashkir Medical Institute (Ufa) and supplied with a photomultiplier tube with a spectral range of 300-600 nm and maximum sensitivity at 400-420 nm. An SKhFM-1 standard with emission intensity of $5.1 \cdot 10^5$ quanta/sec·4π at 470-670 nm was used as a secondary standard.

Horseradish peroxidase (Bioreaktiv, Russia) was dissolved in the above-mentioned buffer without chloram-

0006-2979/01/6603-0282$25.00 ©2001 MAIK "Nauka/Interperiodica"

phenicol (15 units per ml), and luminol (analytical grade, 0.05 mg/ml, Reakhim) was solubilized in twice distilled water.

The measurements were conducted as follows. Peroxidase and luminol solutions (2 ml each) were mixed in a small beaker just before measurements and immediately installed into the operation chamber of the chemiluminometer, which was equipped with a stirrer. The chamber was closed, and after stabilization of the dark current, an aliquot of the analyzed medium was added gradually with a micropipette under constant stirring. Hydrogen peroxide was detected by luminol-induced chemiluminescence. The signal was automatically processed using the computer program supplied by the manufacturer and displayed on the screen as a plot of relative emission units versus time.

Elicitors, a mixture of chitooligosaccharides with molecular masses ranging from 5 to 10 kD and acetylation degree of 65%, were obtained by hydrolysis of chitin with sulfuric acid as described [10]. To induce $H_2O_2$ production, the elicitor dissolved in potassium phosphate buffer was added to seedlings in Petri dishes. Aliquots of the medium were taken after certain intervals with periodic shaking of the dishes, and $H_2O_2$ concentration was immediately measured.

To identify the product inducing chemiluminescence of the medium, in a parallel experiment catalase (Serva, 10 units/ml) was added to an aliquot of the incubation medium after 10 min of incubation with CHOS (1 μg/ml). The results showed that, after 1.5 min of incubation with catalase, chemiluminescence dramatically decreased, and after 3.5 min it was lower than that of potassium phosphate buffer. Upon the addition of inactivated catalase (5 min, 100°C), the chemiluminescence of the medium was at the original level. The amount of $H_2O_2$ in control and experimental variants was expressed in relative chemiluminescence emission units as described by Le Vine et al. [11].

Hydrogen peroxide of analytical grade and known concentration (Reakhim) was purified by repeated distillation and kindly provided by Prof. V. V. Shereshovets (Institute of Organic Chemistry, Ufa Scientific Center, Russian Academy of Sciences). Sodium diethyldithiocarbamate (DTC) and N-acetyl-D-glucosamine (GlcNAc) were obtained from Serva.

The results were obtained in two independent experiments including three biological replicates. The results of one experiment are presented. In the figures mean values obtained in biological replicates are given. Vertical lines show standard deviations.

## RESULTS AND DISCUSSION

As seen from Fig. 1, the height of peaks expressed in relative chemiluminescence emission units positively correlated with $H_2O_2$ concentration. According to the data in the literature, the production of hydrogen peroxide by plant cells is extremely low ($10^{-14}$ M) [12]. Therefore, before measuring $H_2O_2$ concentration in the samples we determined the sensitivity of the instrument and of the developed procedure using standard solutions. For this purpose, we studied the relationship between luminol-induced chemiluminescence emission of $H_2O_2$ solution (0.5 ml) in potassium phosphate buffer and hydrogen peroxide concentration. The minimum $H_2O_2$ concentration estimated with significance as the difference in chemiluminescence of the sample and control (potassium phosphate buffer) was 0.125 μM or about 4 ng/ml. Chemiluminescence of the medium increased with increasing $H_2O_2$ concentration (Fig. 2). A further increase in $H_2O_2$ concentration led to a drastic enhancement of chemiluminescence (at 4 μM $H_2O_2$ it was 19.47 ± 0.27 relative units).

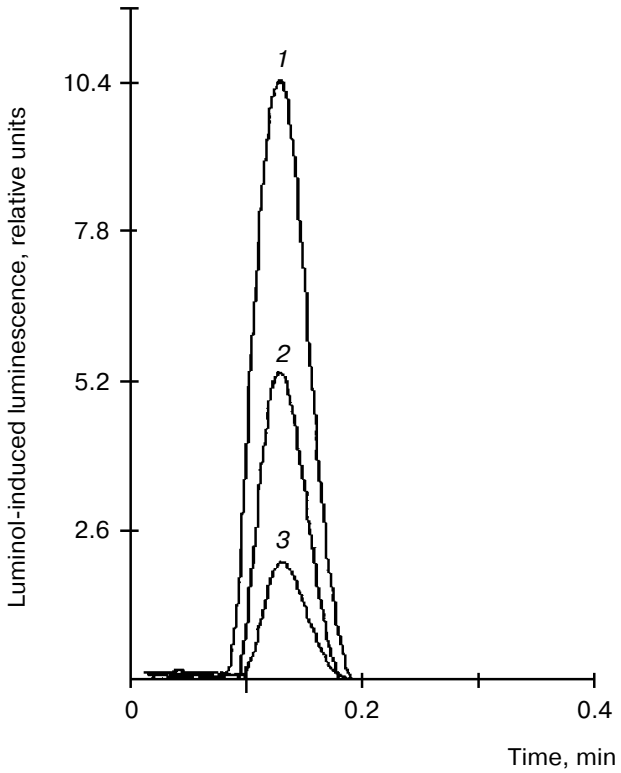


**Fig. 1.** Luminol-induced chemiluminescence recorded on the KhL-003 instrument. $H_2O_2$ concentrations: 2 (*1*), 0.5 (*2*), and 0.125 μM (*3*).

As mentioned above, cell cultures are usually used in studies of hydrogen peroxide production. Works in which intact plants were employed for this purpose are rare. The paper of Dumas et al. [13] attracted our attention. They observed the oxidation of α-naphthol by roots of barley seedlings due to the formation of $H_2O_2$ in the presence of oxalate oxidase. We suggested that roots produce hydrogen peroxide that is released into the growth medium. In the absence of ions with fluctuating valence or oxidases, it

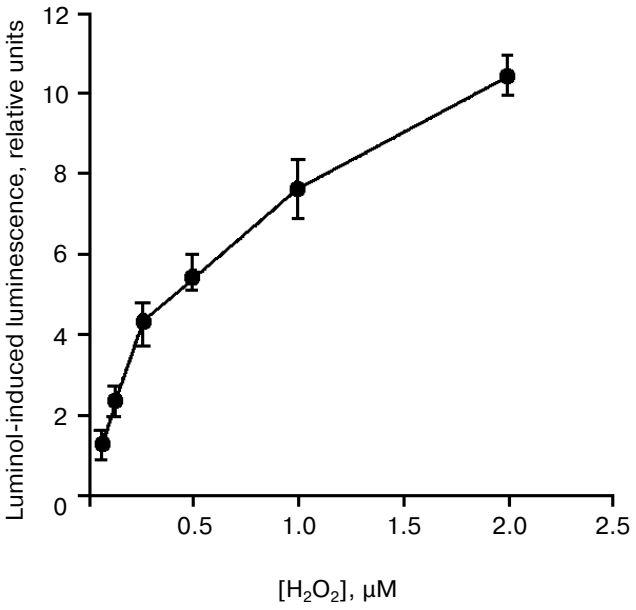


**Fig. 2.** Relationship between chemiluminescence of potassium phosphate buffer and $H_2O_2$ concentration.

seemed possible to measure $H_2O_2$ concentration using chemiluminescence analysis.

Induction of $H_2O_2$ production by chitosan has been reported [14]. In our experiments, we analyzed the ability of CHOS to induce $H_2O_2$ formation in roots of intact wheat seedlings. Preliminary studies showed that CHOS did not cause nonspecific chemiluminescence of the buffer at concentrations below 10 μg/ml.

The results of the experiment are shown in Fig. 3. The addition of CHOS-containing buffer (CHOS concentration of 1 μg/ml) to the roots of seedlings led to a considerable increase in luminol-induced luminescence as compared to the control (potassium phosphate buffer). As seen from this figure, the maximum emission was observed after 30-40 min of incubation with the elicitors.

The biological effect of chitooligosacharides on plant cells depends on the length of oligomers, the degree of acetylation, and the presence of functional groups [15]. Therefore, we used the chitin monomer GlcNAc (10 μg/ml) for comparison. As seen from Fig. 3, even at concentrations exceeding those of CHOS, GlcNAC did not stimulate $H_2O_2$ formation; this indicates a specific reaction of plants to CHOS. The induction level depended on CHOS

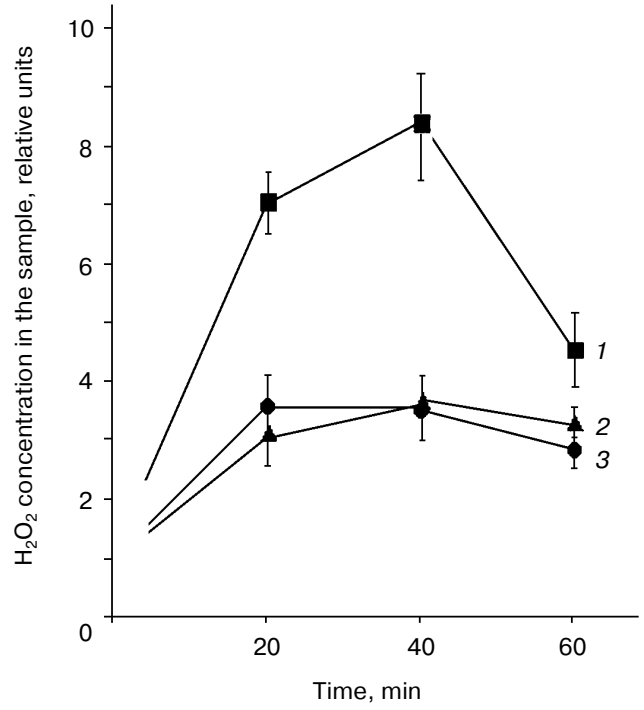


**Fig. 3.** Overproduction of hydrogen peroxide by wheat seedlings induced by chitooligosaccharides (CHOS). To the seedlings growing on potassium phosphate buffer, the following components were added: CHOS (1 μg/ml) (*1*), GlcNAc (10 μg/ml) (*2*), or potassium phosphate buffer (*3*).

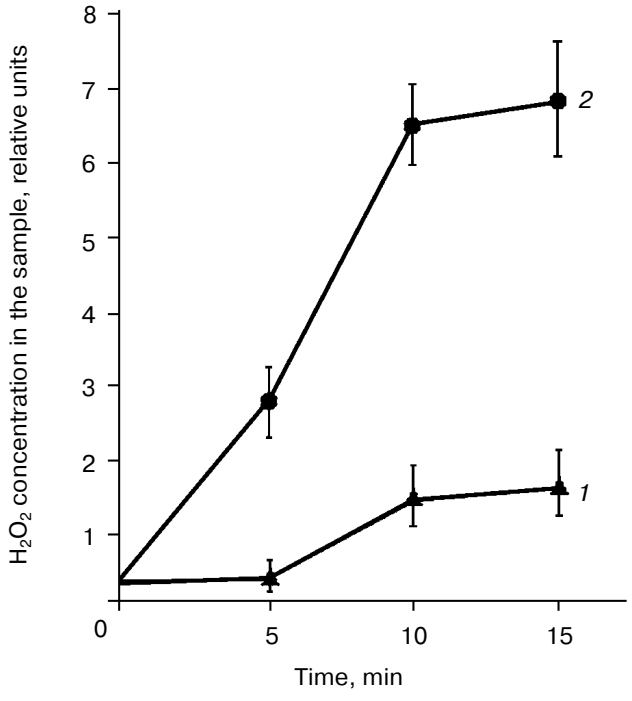


**Fig. 4.** Effect of DTC (*1*) on $H_2O_2$ production. In the control (*2*), seedlings were placed in potassium phosphate buffer and after 10 min of incubation treated with elicitors (1 μg/ml). In the experimental variant, the incubation buffer contained 10 mM DTC; other conditions were the same.

concentration in the medium and the time of incubation. The minimum concentration increasing $H_2O_2$ production after 10 min of incubation was 10 ng/ml.

To exclude the possibility that $H_2O_2$ formation was due to microorganisms growing on roots, we recovered sterile plants from sterilized seeds on Chapek agar (Reakhim). Seedlings that grew on the medium without bacterial colonies were examined. In a laminar-flow hood under sterile conditions, seedlings were prepared for experimentation. However, even in this case CHOS induced $H_2O_2$ production, indicating its plant origin.

The formation of hydrogen peroxide in animal and plant cells is associated with the conversion of a superoxide anion radical $O_2^{\cdot-}$. To elucidate the mechanisms of $H_2O_2$ overproduction after treatment with CHOS, we used DTC, which inhibits $O_2^{\cdot-}$ dismutation [16]. Before treatment with elicitors, plants were incubated for 10 min in DTC-containing medium (the DTC concentration was 1 mM) in Petri dishes. Aliquots of the buffer (control) or CHOS solution (1 μg/ml) were added to the medium. As seen from Fig. 4, treatment of plants with DTC greatly inhibited $H_2O_2$ production as compared to control. However, CHOS-induced formation of $H_2O_2$ still occurred with time, but it was less pronounced than in control plants.

Our results indicate that the KhL-003 instrument can be used for chemiluminescence analysis of $H_2O_2$ production at concentrations above 0.125 μM. As we have already mentioned, cell cultures are commonly used in physiological or biochemical studies of production of active oxygen species. We have shown that intact plants can serve as convenient models. Using wheat seedlings, we have demonstrated that chitooligosaccharides exhibit high biological activity inducing overproduction of hydrogen peroxide at a concentration of 10 ng/ml.

Analysis of intact plants has several advantages over cell cultures: the experimental procedures are much easier and provide more reliable information, since the reaction to external stimuli of isolated cells lacking their natural environment and of the intact plants may be quite different.

## REFERENCES


1. Doke, N. (1983) *Physiol. Plant Pathol.*, **23**, 345-357.
2. Apostol, I., Heinstein, P. F., and Low, P. S. (1989) *Plant Physiol.*, **90**, 109-116.
3. Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. (1994) *Cell*, **79**, 583-593.
4. Wu, G., Shortt, B. J., Lowrence, E. B., Levine, E. B., Fitzsimmons, K. C., and Shah, D. M. (1995) *Plant Cell*, **7**, 1357-1368.
5. Dixon, R. A., and Harrison, M. J. (1994) *Annu. Rev. Phytopathol.*, **32**, 479-501.
6. Davis, D., Merida, J., Legendre, L., Low P. S., and Heinstein, P. (1993) *Phytochemistry*, **32**, 607-611.
7. Kurosaki, F., Tashiro, N., and Nishi, A. (1996) *Plant Cell Physiol.*, **27**, 1587-1591.
8. Vander, P., Varum, K. M., Domard, A., El Gueddari, N. E., and Moerschbacher, B. M. (1998) *Plant Physiol.*, **118**, 1353-1359.
9. Natsu, Sh., Saito, N., and Kosaki, H. (1994) *Biosci. Biotech. Biochem.*, **58**, 552-553.
10. Akhmetova, I. E., Mullagaliev, I. R., Akhmetov, R. R., and Khairullin, R. M. (1998) in *Proc. of the Biological Department of Bashkir State University* (Akhmetov, R. R., ed.) [in Russian], Bashkir State University, Ufa, pp. 68-72.
11. Le Vine, D., Kaplan, M. J., and Greenaway, P. J. (1972) *Biochem. J.*, **129**, 847-856.
12. Keppler, L. D., Baker, C. J., and Atkinson, M. M. (1989) *Phytopathology*, **79**, 974-978.
13. Dumas, B., Freyssinet, G., and Pallet, K. (1995) *Plant Physiol.*, **107**, 1091-1096.
14. Legedre, L., Reuter, S., Heistein, P. F., and Low, P. S. (1993) *Plant Physiol.*, **102**, 233-240.
15. Doares, S. H., Syrovets, T., Weiler, E. W., and Ryan, G. A. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 4095-4098.
16. Jabs, T., Tschope, M., Colling, C., Hahlbrock, K., and Scheel, D. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 4800-4805.