Express Control of Toxicity and Content of Patulin by Optical Biosensors

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ABSTRACT

The main principles of the determination of patulin toxicity (bioluminescent test with bacteria and Daphnia magna) as well as quantitative control of this mycotoxin by SPR based immune biosensor with different algorithms of analysis are presented.

Keywords: patulin, optical biosensors, determination of toxicity, quantitative control

1 INTRODUCTION

Today it is known over 500 toxic metabolites produced by more than 250 strains of microscopic fungi's. The development of the investigations in the field of mycotoxicology has shown that the intoxication by mycotoxins is not only real but very serious dangers for people. Moreover, the chronic mycotoxicosis significantly overcomes all known events of the acute forms of the poisons in Japan, Russia, India, China and USA with the lethal way out for many thousand people [1]. Among of mycotoxins which are as the etiological factor for the number of nutritional toxicosis and which are very widespread a special place belongs to patulin (P) presented by lacton(4-hydroxi-4*H*-furo[3,2-*c*]pyran-2-(6*H*)-one) with molecular mass in 154,12 and empiric formula $C_7H_6O_4$. Firstly P was obtained in 1943 from the culture of fungi of *Penicillium patulum* (synonym of *Penicillium urticae*) as antibiotic and at the beginning it was considered as non-dangerous mycotoxin. Nevertheless in 1954 it was as the reason for dead of 100 cows in Japan. Different strains of fungi's may be as producers of P, for example: *Penicillium*: *P. expansum, P. claviforme, P. urticae* (*P. patulum*), *P. cyclopium, P. viridicatum, P. roqueforti*; as well as strain of *Aspergillus: A. clavatus, A. terreus, A. giganteus*. *Byssochlamys fulva, Byssochlamys nivea, P. mortensic* and *Paecilomyces* may produce P too. Nevertheless, the fungi's of *P. expansum* strain are as stronger P producer. Revealed high level toxicity, mutagenic and cancerigenic abilities in P were as a basis for the JECFA to restrict the maximal permissible dose of the P incoming in organism on the level of 0.4 ng/kg of body mass per day [2]. National and International groups are recommended to use apple foods in which the residual content of P is no more than 50 μg/kg, at the same time many countries regulate the level of this mycotoxin in the juice on the level from 20 to 50 μg/kg.

The different types of the chromatography: one- and two-dimensional thin-layer chromatography – TLCh (at the serial analysis), high performance liquid chromatography (HPLC) with ultraviolet- and fluorometric detection (at the serial and control analysis) are used for the revealing, identification and quantitative determination of P. Review of the methods of the P determination shows that the TLCh methods are more difficult and provide less precision and sensitive analysis in the comparison with the HPLC [1]. Isotopic analysis allows the determination of this mycotoxin at the concentration of 12 $\mu g/dm^3$.

P and others mycotoxins are low molecular weight substances due to they may be as antigens but not as immunogens. The last ability they may have after conjugation with some proteins. As a rule it is not simple procedure and demands often high professionalism of chemists. It was informed about obtaining of the polyclonal antibodies to P although their specificity is not high. Unfortunately, the commercial monoclonal antibodies to this mycotoxin were not revealed in any firm cataloques in spite of a big interest of practice to the development of the different types of the immunochemical analysis including its instrumental, in particular, biosensoric variants. Today's demands of practice to the methods of the quantitative food control have grown very sharply. They include the number of positions, namely: the high level of the selectivity and sensitivity of analysis, its rapidity and low cost, possibility of the fulfillment of the observations in the field conditions and in regimes of line and on line. Moreover, it is very important to check simultaneous several samples from the same or from the different sources and, at last, the providing of electronic processing of the obtained results and their automatic transferring in the special laboratories or in the controlling offices. The fulfillment of all these demands is possible with the development and production of the principal new instrumental analytical devices. Biosensors may be classified as such perspective devices. They most complete respond to the all complex of the strong practice demands not only in field of biotechnology and in medical diagnostics and ecological monitoring [3-8]. To that time the immune biosensors were worked out for the determination of low molecular weight substances in water and foods. It concerns heavy metal ions, phosphororganic pesticides, atrazin, simazin, nonylphenol, T2 micotoxin, aflatoxin, deoxinivalenol and others [9-11].

It is very pity but we have not found any information about the development of biosensors for the control of P.

The main purpose of this article is the presentation of our experimental results about the determination of P toxicity and the development of both the ELISA-method and the immune biosensor based on the surface plasmon resonance for control of this toxin level in environmental objects. At the same time it was planed to solve a task connected with the preparation of the needed immune components and working out of the main algorithm of analysis at the use of the model P solutions.

1. EXPERIMENTAL

Toxicity of P was characterized by optical biosensors based on the determination of the chemiluminescence (ChL) of *Daphnia magna* living media (ChL-test) and by the bioluminescence (BL) of bacteria (BL-test) as it was described early [12, 13]. To register biochemical signal the special stationary and portable devices were developed.

From the steric structure of P (Fig. 1) it can see that for the preservation of the antigen image of this mycotoxin there is necessary to modify some chemical groups before the use of them in the process of conjugation with the proteins. The all needed modifications of P and its conjugation with bovine serum albumin (BSA) and horse radish peroxidase (HRP) (both from Sigma, USA) were accomplished according to [14]**.** To obtain the specific antiserums rabbits were immobilized according to next scheme. First immunization was accomplished by the intracutaneously injection of the mixture contented the solution of P-conjugate (in isotonic NaCl) and complete Friends adjuvant (Calbiochem, USA). Through 3 weeks this procedure was repeated. In next period the immunization cycle was finished by two injections with the interval of 2 weeks but without use of Friends adjuvant. The re-immunization was fulfilled through 1 month. Blood was taken trough 12 days (variant of 1), after two injections of antigen (variant of 2) and trough 2 weeks after re-immunization (variant of 3). Total fraction of immunoglobulin (Ig) was obtained by well-known procedure with the application of sulphate ammonia precipitation.

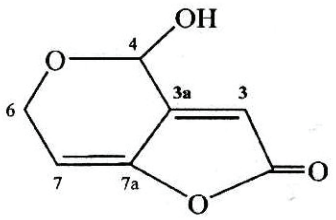

Figure 1. Steric patuline structure.

The ELISA-method was used for the quantitative determination of P and it was curried out in two variants. In both variants free P competed with one of its conjugate for the binding sites of the antibodies immobilized on the plate surface. Diapason of the free and conjugated P concentrations has varied in frame of 0.1 ng/mL – 100 μg/mL (in the ratio 1:1). As a rule 100 μL of BSA-P conjugate at the concentration of 20 μg/mL in 0.1 M sodium phosphate buffer, pH 8,2 were introduced into cells of the immunological plate and for 18 h at +4°C. After that the cells were washed 3 times by 0,1 M sodium phosphate buffer (pH 7,4) contained 0,14 M NaCl (PBS). To block free binding places 150 μL of gelatin solution at the concentration of 0.1% were placed into cells and they were incubated during 30 min at +37°C. Then cells were washed by PBS contained twin-20 (0.05%) and filled by 50 μL of the solution of P specific antiserum (preliminary dissolved by PBS with 0,05% of gelatin) and 50 μL of free P solution (at the different concentration in methanol-water medium in ration 1:4). This mixture was incubated during 1 h at +37°C. At last, cells were washed by PBS and filled by the solution of goat anti-rabbit antibodies conjugated with HRP which was preliminary diluted by PBS in the ratio 1:1000. Through 1 h of incubation at +37°C the cells were washed 3 times by PBS and 1 time by the distilled water. Activity of HRP was determined in the presence of ortophenylendiamine and H_2O_2 (1,8 мМ) in 30 мМ sodium acetate buffer, pH 4.5. Optical density of the solution was measured through 30 min with the help of "Sumal PE-2" device (Germany). The obtained results were presented in the values of optical units (O.U). Both variants had only one difference; in particular, it was connected with the application of P-HRP conjugates which excluded the necessity to use the goat anti-rabbit antibodies conjugated with HRP.

Immune sensor analysis carried out on the device SPR-4M made in the Semiconductor Physics Institute (Ukraine). Forty-manometer-thick gold film was prepared by vapour deposition of metal on the glass surface of a prism. In the experiments gold surface was modified with dodecanthiol (during 12 h) or polyalylamine chloride to prevent protein destruction. The analysis was fulfilled in several ways: direct, competitive and so called “to saturation”.

The “direct” way was the most simple and its fulfilment was carried out in the following way. At first the initial SPR angular profile for PBS was recorded and the SPR angle corresponding to the minimum reflection intensity was determined. Then the appropriate solutions of the P-conjugate or total IgG specified to this mycotoxin (both in the concentration of 1 mg/mL) were introduced into the measuring cell (volume 10 μL). The adsorption of the above mentioned substances occurred at room temperature over a period of 30 min. The measuring cell was then rinsed with PBS and filled with BSA solution (1%) to prevent non-specific binding of specific immune components to the transducer surface. After the incubation period of 10 min the measuring cell was washed with PBS and new value of SPR angular profile was recorded. It served as reference line. Then, the measuring cell was filled with the P solution with the appropriate concentration. The incubation time was equal to 10 min, which allowed angle profile to reach

plateau. After this the measuring cell was washed with PBS contained 0.1% of Tween-20 to remove all unbound components from the surface layer, preventing thus any interference with the measurement of the SPR angle caused by these free analyte molecules in solution. "Competitive" way was fulfilled in two variants. Both of them were similar as the above described one but in this case the P-conjugate or specific total Ig were immobilized in the measuring cell. Then it was filled by the mixture of total IgG specified to P and free P, or by free P and its conjugate at the following concentration: 1, 10, 100, 1000 ng/mL and 1, 10, 100 μg/mL. In both cases free and conjugated molecules of P competed for the binding centres of the specific Ig. "To saturation" way had some difference in that the specific Ig was immobilized in the surface of the measuring cell and then they reacted with free molecules of P. The reminded centres of the specific Ig were saturated by the P-conjugate.

There is necessary to underline that in all cases when the specific Ig were immobilized on the surface of measuring cell the intermediate layer between them and dodecanthiol or polyalylamine sulphate was formed from the protein A. It allows orienting of the binding centres of Fab-fragments of the specific Ig toward solution.

To determine P in real samples it was the fulfilment of the procedure of their preliminary treatment according to [1]. Tomate juce was used as model of real sample in which a different concentration of P were added.

3 RESULTS AND DISCUSSION

The preliminary obtained results shown that P belongs to strong toxic substances. In the next investigations it was revealed concrete details of this toxicity. The increase of P concentration from 0.63 to 40 mg/L leads to sufficient decrease of BL during 12-60 min of its influence on the *Phosphorescenum phosphoreum Sq3* bacteria. The value of effective toxic concentration (ETC) is in range of 0.63-1.25 mg/L. With the prolongation of P effect on bacteria this value is in frame of 0.15-0.63 mg/L . At the decreases of medium pH up to lover physiological level (5-5.5) the bacteria sensitivity to P the bacteria sensitivity increased almost on one order and it was equal to semi-lethal dose established in experiments with animals [15]. In example it was shown that destroying of intestinal barrier was observed at the P concentration in apple juice on the level of 1 mg/kg. Bacteria of *Vibrio fishery* had more high level sensitivity in comparison with the bacteria *Ph. phosphoreum Sq3* keeping the same effect connected with the lowering of medium pH.

The sensitivity of standard international method based on *D. magna* to sodium bichromate was about 0.9-2.0 mg/L. At the time ChL-test was more sensitive up to 3 orders. It is necessary to underline that developed instrumental approach demands 30 min only in comparison with several days needed for standard procedure. P in concentration from 0.01-1 mg/L aroused adaptive *D. magna* reaction.

At the increase of P concentration higher 1 mg/L it was observed sharply decrease of ChL signal. Maybe in first case P stimulates stomach of *D magna* system to excrete some substances which are able to stimulate ChL. In other one P aroused daphnia mortality. It is necessary to mentioned that both proposed optical biosensors are characterized by high sensitivity and they can fulfill all practice demands in the respect of estimation of total toxicity of environmental objects before the solving of the question: is it needed to check them on the presence of some groups of toxic elements or concrete toxins.

The titer of antiserum was checked by the traditional method of the immune precipitation. It was stated that it was similar for both conjugates and achieved level up to 1:64 in case of the immunization according to variant 3 (see details above). Certainly at the use of variants 1and 2 the obtained level of the titer was much high (in two and three times, respectively). It was stated that all antiserums are characterized by the high level of the specificity to P. That is why there is basis to hope that they may provide specific determination of this mycotoxin in samples to be analyzed.

Calibration curves for P determination were obtained with the application of both types of the specific antiserum. It was shown that the both antiserums allow revealing P in the diapason of concentrations from 10 to 1000 ng/mL. The purified total fractions of Ig have shown the same calibration curves as it was found for the appropriate antiserums. The observed difference between the antiserums is not sufficient for the analysis and it may be connected with the differences in the concentration of the specific antibodies in the serum blood. Both calibration curves were plotted at the initial dissolving of the antiserums in ratio 1:500. At the decreasing of this ratio up to 1:300 the early observed difference eliminated.

"Direct" analysis by SPR immune biosensor allows to us revealing P at the concentration of 1 mg/L (Fig. 1A). It is not practically significant level. The "competitive" variant was much more sensitive (up to 2 orders). It can provide the determination of P at the concentration in the range from 0.01 to 10 mg/L (Fig. 1B). The similar results were obtained at the realization of "to saturate" way at the P determination. There is necessary to underline that the comparison of the application of polyelectrolites and dodecanthiol demonstrates the advantages of first intermediate layer to second one. Mainly it is connected with the stability of results between measurements. The results of P determination in real samples (tomato juice) by "competitive" way are presented in Fig. 2. It can see that we have possibility to reveal P at the concentration starting from 0.05 mg/L. The linear diapason is in range of 0.05-10 mg/L. We think that the difference which is observed between standard solution and real sample is stipulated by some non-reversible sorption of P in organic phase. The next experiments with different products give possibility to optimize procedure of extracton.

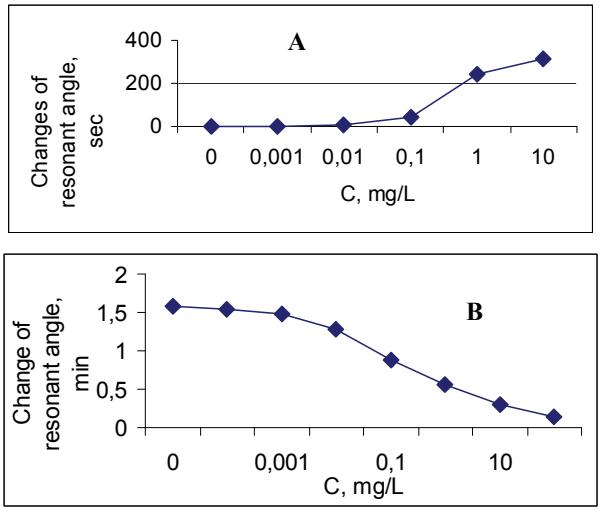

Figure 1. Calibration curve for the P determination by SPR immune biosensor at the direct (A) and "competitive" (B) analysis.

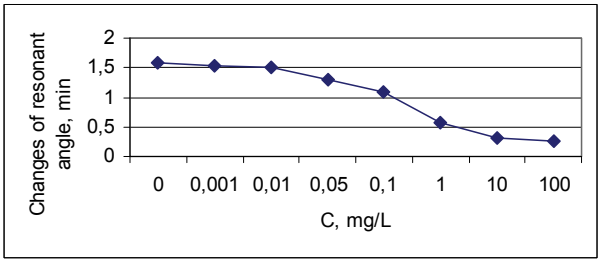

Figure 2. Determination of P in tomato juice by SPR immune biosensor at the "competitive" analysis.

4 CONCLUSION

The developed optical biosensor systems are able to provide all practice demand in the respect of screening environmental objects on total toxicity which may be stipulated by the P presence and concerning to determine concrete concentration of this toxin.

ACKNOWLEDGMENT

This work was partially supported by State Fond of the Fundamental Investigations in Ukraine, project N F28.7/020.

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