Ildikó Barna-Vetró[1], László Solti[2] and Ervin Balázs[1]*

Immundiagnostics Serving Food Security

Introduction

Recent highly publicised issues on dioxin contamination of feed in Belgium, the BSE epidemy in the UK or the illegal hormonal treatment of swine in Bavaria underlines the importance of food and feed safety. Since the inauguration of Agricultural Biotechnology Center at Gödöllő, in 1990 food safety related R&D has priority in the Center's research portfolio. The Diagnostic Laboratory of the Center established right at the beginning of the research work has initiated a long term research programme for development of monoclonal antibody based ELISA kits along with the assay technology. Food and feed industry requires sensitive and reliable detection systems for mycotoxin and antibiotics contamination at an acceptable cost level.

Only in the last 30 years has it become clear that commonly occurring fungi growing in foods and feeds may produce toxins, known as mycotoxins. Mycotoxins are secondary metabolites of fungi, some of them have molecules with structures ranging from single heterocyclic rings to complex six or eight rings. Under our continental climate, the most frequent *Fusarium* toxins are ***T-2, zearalenone (F-2), DON, DAS*** and ***fumonisins (FB_{1-4})***, originating from infected cereals either on the field or during storage. By consumption of these cereals the mycotoxins enter the organisms of humans and animals. The symptoms of mycotoxicoses are as diverse as the chemical structures of the compounds themselves such as: feed refusal, nervous system disturbances, diarrhoea, irregular or absent oestrous cycles, decreased milk production, leukoencephalomalacia in horses, pulmonary oedema in pigs etc. (1, 2).

Ochratoxin-A produced by some *Aspergillus or Penicillium genera* is a proven teratogenic and potentially carcinogenic compound causing maternal toxicosis, decreased foetal weight, and chronic renal disease (2). Although the temperate climate in Middle Europe is not favourable for growth of strains of *Aspergillus* moulds, their carciogenic metabolites called ***aflatoxins*** can cause serious health problems by consumption of imported foods and feeds.

Recent interest in these toxins has led to propose a legislation concerning „acceptable limits". The legitimate mycotoxin limits according to the Hungarian Mycotoxin Standard are shown in *Table 1*. These regulatory limits are in accordance with those of the European Union or even more strict. In order to enforce these limits, reliable methods of detection and determination are required. Since enzyme-immunoassay is inexpensive and fulfils the requirements of sensitivity, simplicity and reliability, ELISA tests based on monoclonal antibodies were developed for mycotoxin analysis. As a result of our R+D activity five mycotoxin kits (T-2, F-2, ochratoxin-A, fumonisin B_1 and aflatoxin B_1) have already been developed, branded under the name of TOXIKLON and are now commercially available. Here, we report a general overview about our research achievements.

Table 1. *Acceptable limits for animal feed products in Hungary*

Mycotoxins	Animal feeds for	Regulatory limit ($\mu g.kg^{-1}$)
Zearalenone	raw materials	10000
	grown-up ruminants	2000
	breeding (cattle, swine, turkey)	80
	laying hen, broilers, swine	500
T-2/HT-2	grown-up ruminants	1000
	laying hen, broilers, swine	300
Deoxynivalenol (DON)	grown-up ruminants	2000
	breeding (cattle, swine, turkey)	400
	laying hen, broilers, swine	2000
Ochratoxin-A	grown-up ruminants	100
	laying hen, broilers, swine	10
	other feeds	25
Aflatoxin B_1	raw materials	50
	breeding (cattle, swine, turkey)	5
	laying hen, broilers, swine	20
	other feeds	10

Our immunoassay know-how

Immunoassays are of growing importance as a screening tool in mycotoxin analysis. All immunochemical methods are based on binding properties of the analytic with its specific antibody. The quality of immunoassays depends primarily on the quality (affinity, avidity, cross-reactivity) of the antibody. Using the appropriate

[1] *Agricultural Biotechnology Center, P. O. Box 411, H-2101 Gödöllő, Hungary*
[2] *University of Veterinary Science, P. O. Box 2, H-1400 Budapest, Hungary*

Table 2. *Specification of the monoclonal antibodies*

Monoclonal Antibody	Mycotoxin metabolites	Cross-reactivity (%)	IC_{50} (ppb)	Affinity constant (lxM^{-1})
anti-T-2 (clone No.: 8)	T-2 acetyl T-2 HT-2 Iso T-2	100 12.3 3.4 2.5	2.3	3.3×10^{10}
Anti-F-2 (clone No.: 88)	zearalenone zearalenone α-zearalanol β-zearalanol α-zearalenol β-zearalenol	100 138 69 6 91 21	0.6	7.5×10^{10}
Anti-OA (clone No.: 5G4/4A4H)	ochratoxin-A ochratoxin-B	100 9.3	0.45	7.8×10^{9}
anti-fumonisin B_1 (clone No.: 1D6/F11E3)	FB_1 FB_2 FB_3 HFB_1	100 91.8 209 0	5.4	1.3×10^{10}
anti-aflatoxin B_1 (clone No.: 6G4F7/F3)	aflatoxin B_1 aflatoxin B_2 aflatoxin M_1 aflatoxin M_2 aflatoxin G_1 aflatoxin G_2	100 76 79 33 55 6	0.1	5.6×10^{9}

IC_{50}: 50% displacement value (ppb)

immunogen, immunization protocol, and screening procedures high-quality antibodies can be obtained. As the molecular weight of mycotoxins is generally less than 1000 Dalton, these so called haptens had to be coupled to large carrier molecules, such as proteins (BSA or KLH) in order to become immunogenic. Using the advantage of the hybridoma technology for yielding antibodies with appropriate affinity and specificity, we prepared a series of monoclonal antibodies with high affinity (T-2, zearalenone, ochratoxin-A, aflatoxins B_1 and M_1, fumonisin B_1). The first steps in developing a direct competitive ELISA are to determine the working dilution of the antibody, the peroxidase labelled conjugate, the optimal reaction volume, time and temperature. Monoclonal antibodies were used as ascites fluid without purification, because they did not cause any non-specific reaction in the tests. *Fig. 1.* shows typical dose-response curves of our monoclonal antibodies in buffer solution. The 50% displacement values (IC_{50}), the cross-reactivity with related mycotoxin metabolites and the affinity constants of the antibodies are summarized in *Table 2.* The within-assay and interassay coefficients of variation for the mycotoxin standard concentration of the ELISA tests were always < 10%. Determination of the matrix effects, which in most cases can be avoided by cleaning the samples is one of the crucial parts of the developing work. To extract mycotoxins from different cereals, methanol- or acetonitrile-based organic solvents are generally used in various concentrations, combined with different cleanup steps. The aim was to eliminate sample cleanup prior to the assay in order to simplify the procedure. Solvents containing acetonitrile in different concentrations, proved to be suitable for T-2, F-2, aflatoxin B_1 and fumonisin B_1 extraction from cereals (wheat, barley, maize, soya, rice etc.) (4,7). The extract remained clear, and by appropriate dilution of the samples with PBS-Tween, the interference could be minimized. The composition of the extraction solvent, used for OA from different matrices was different from any other methods published elsewhere. The solvent used contains dichloromethane and citric acid instead of other strong acids (HCl, acetic acid, phosphoric acid). Experiments proved that this mild acid is as effective as strong acids used by others. Centrifugation was one of the most crucial steps because care must be taken to avoid the non-specific reaction caused by incomplete separation of aqueous buffer solution from the organic phase (3, 5, 6). Our ochratoxin test was applied for quantitative measurement of OA in 355 human sera. According to this survey the OA concentrations of serum samples varied from < 0.2 to 10 ng/mL OA, however, 75% of the samples contained

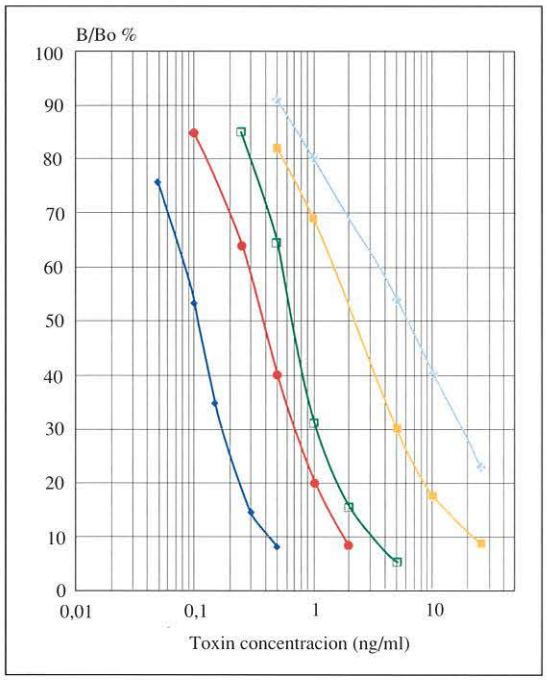

Figure 1. *Dose-response curves of monoclonal antibodies against*
-◆- aflatoxin B_1, -●- ochratoxin-A, -□- zearalenone, -■- T-2, -✧- Fumonisin B_1

Table 3. Specification of Toxiklon kits

TOXIKLON KIT	Detection limit (ppb)	Sample preparation (hour)	Test time (hour)	Assay range (ppb)	Recovery rate (%)
T-2	50	3.5	1.5	100–2000	85
Zearalenone	25	3.5	1.5	50–400	91
Ochratoxin-A	0.5	4.0	1.5	1–10	97
Fumonisin B_1	7.6	4.0	1.5	10–500	84
Aflatoxin B_1	0.9	3.5	1.5	1.5–15	80

0.2–1.0 ng/mL, which amount the organism still able to „tolerate". In some cases (6.8%), more than 1.0 ng/mL OA was measured, which is probably a result of elevated OA intake exceeding the „virtually safe dose". Data indicate that, like in many countries, OA is present in food or feed products, thus, in order to save the health of consumers, their regular control is desirable (5). In a recent paper we investigated whether the intake of high dose of OA has any effect to the motility and longevity of boar spermatozoa. Our preliminary results suggest that OA might have the potential to affect sperm production and semen quality of boars (6).

For easier application of our ELISA tests in practice, reagent kit-packages have been set up. The brand name of these commercialised kits is TOXIKLON (*Fig. 2.*). Specifications of the kits are summarized in *Table 3*. Each kit is sufficient for quantitative analysis of 40 cereal samples in duplicates. The kits are flexible and by using strip format microplates, individual samples can also be determined. For longer shelf-life, conjugates are in a stabilizing buffer, all the other solutions in concentrated form; the stability of these reagents is thereby guaranteed for 6 months. Our kits are used mainly in Accredited Food and Feed Control Laboratories in Hungary for rapid screening of mycotoxins. Some selected samples, containing mycotoxins around the regulatory limit or above have been confirmed by accepted analytical methods (HPLC, GC-MS).

Our laboratory has obtained ***ISO 9001:2001*** qualifications for production of monoclonal antibodies against mycotoxins and antibiotics and for the development of ELISA test-kits for mycotoxins and antibiotics.

The Diagnostic Laboratory has scientific contracts with institutions including University of Ghent and Universities of Helsinki and Kuopio. The Center signed licence contracts with Romer and EnviroLogix (USA) and also with Eurodiagnostic (The Netherlands) firms for the use of our monoclonal antibodies and mycotoxin-peroxidase conjugates in their own developed diagnostic systems.

Details on the immundiagnostic program can be seen on the following website: www.abc.hu

Figure 2. *TOXIKLON ELISA kits for mycotoxin determination*

References

1. D'Mello, J. P. F., Placinta, C. M. and **Macdonald, A. M. C.** (1999): Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity. Animal Feed Science and Technology. 80. 183–205.

2. Pitt, J. I., Basilico, J. C., Abarca, M. L. and **López, C.** (2000) Mycotoxins and toxigenic fungi. Medical Mycology 38. 41–46.

3. Barna-Vetró, I., Solti, L., Téren, J., Gyöngyösi, Á., Szabó, E., Wölfling, A. (1996) „Sensitive ELISA test for determination of ochratoxin A." J. Agric. Food Chem., 44, 4071–4074.

4. Barna-Vetró, I., Solti, L., Szabó, E. and **Gyöngyösi, Á.,** (1997) Sensitive enzyme-linked immunosorbent assay for screening aflatoxin B1 in food – and feedstuffs, Cereal Research Communications 25, 291–292.

5. Solti, L., Salamon, F., Barna-Vetró, I., Gyöngyösi, Á., Szabó, E. and **Wölfling, A.,** (1997) Ochratoxin A content of human sera determined by a sensitive ELISA, J. Anal. Toxicol., 21, 44–48.

6. Solti, L., Pécsi, T., Barna-Vetró, I., Szász (Jr), F., Biró, K., Szabó, E., (1999) Analysis of serum and seminal plasma after feeding ochratoxin A with breeding boars, Anim. Reprod. Sci., 56, 123–132.

7. Barna-Vetró, I., Szabó, E., Fazekas, B. and **Solti, L.** (2000) Development of a sensitive ELISA for the determination of Fumonisin B1 in cereals. J. Agric. Food Chem. 48, 2821–2825.

* Corresponding author. Agricultural Biotechnology Center,
H-2101 Gödöllő, P. O. Box. 411.
Phone: +36-28-430-600;
Fax: +36-28-430-647.
E-mail: barna@abc.hu