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Magnetic ELISA of aflatoxin B1 - pre-concentration without elution

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While immunoenzyme assay (ELISA) is widely used for detection of various compounds, its use is significantly limited by the considerable duration (determined by the heterogeneous reaction to form detectable immune complexes) and the restricted detection limit. This study proposes an ELISA variant based on the application of highly dispersed (average diameter ~ 10 nm) magnetic iron oxide nanoparticles as carriers for the adsorbable antibodies. In conducting the proposed ELISA, the antibodies react with the detectable compound within the sample volume; the formed complexes are preconcentrated by precipitation in a magnetic field and are used for immunoenzyme detection in the wells of a microplate. This approach has been implemented for detection of aflatoxin B1, a low molecular weight compound that needs to be controlled at extremely low concentrations due to its high toxicity. Using magnetic nanoparticles provided a 10-fold lowering of the detection limit and cut the test duration in half, compared to conventional ELISA. Immobilized antibodies exhibited high resistance to methanol when testing aqueous/methanol extracts of contaminated vegetable feed stocks (corn kernels), making it possible to identify aflatoxin B1 at concentrations as low as 2 pg/ml (40 pg/g). The proposed approach is universal and can be used for immunodetection of various compounds.

1. Introduction

2. Immunoenzyme assay (ELISA) is one of the most widely used analytical approaches in modern medicine, environmental monitoring, quality control, and food safety. Its unquestionable benefits include simplicity, high throughput, reproducibility, and low cost of testing.

3. However, the conventional ELISA protocol prevents the lowering of detectable concentrations of the target compounds and reduction in analysis time. The detection limit in most immunoenzyme test systems is controlled by the equilibrium constant of the antigen-antibody reaction and is limited by the upper limit value of this constant, determined by the immune response induction mechanism.

4. The reason for the extended (several hours) duration of solid phase ELISA is the heterogeneous interaction between immunoreactants in solution and those immobilized on the surface of the carrier. Slow diffusion exchange between liquid layers positioned at different distances from the carrier hinders the reduction of analysis time. In contrast to solid phase ELISA, homogeneous immunoassay systems lack this disadvantage; however, the immune complexes formed therein are not separated from the unreacted molecules, and therefore, highly sensitive detection of the analyte is complicated.

5. In view of the advantages and disadvantages of homogeneous and heterogeneous immunoassay methods, it seems appropriate to combine in a single scheme the rapid formation of immune complexes in solution with their detection effectiveness in heterogeneous systems.

6. The use of highly dispersed carriers provides the ability to increase the surface area for immobilization of reactants and to distribute them uniformity throughout the volume of the reaction medium, thereby speeding up the heterogeneous interactions. Such work is being carried out in several directions.

7. One of the directions involves the use of charged carriers or polyelectrolytes in immunoassay. Having obtained the conjugate of one of the polyelectrolytes with immunoreactant, the target antigen may be detected in solution just as in conventional homogeneous methods; the formed immune complexes can then be rapidly separated by using a counter-ion or another reagent that would precipitate the polymeric carrier. The second option involves the use of a suspension of ultradispersed immunosorbent separated by centrifuging after the specific reaction. For example, the immunosorbent carrier may be polystyrene particles, allowing the immobilization of antibodies by adsorption, as in conventional ELISA.

8. However, centrifuging becomes time-consuming when performing a large number of parallel analyses, and it requires specially adapted equipment. In this respect, the third direction, involving the use of magnetic immunosorbents, appears more promising. Application of a magnetic field after the immunochemical interactions provides a very simple and rapid means of separation of the reactants. This separation is achieved by using holders with permanent magnets of a certain size and strength; it...
2.2. MNP Synthesis

33 30% ammonium hydroxide was added dropwise to a
34 148 concentration of 2.4% to an aqueous solution containing
35 149 3.4 mg/ml FeCl2 and 3.6 mg/ml FeCl3. The mixture was
36 150 incubated for 15 minutes at room temperature with
37 151 vigorous stirring Intelli-mixer RM-2 (Elmi Ltd., Latvia,
38 152 www.elmi-tech.com). The chosen rotation mode was F1 in
39 153 accordance with 70 rpm. The particles were precipitated
40 154 by applying a magnetic field and after removing the
41 155 supernatant, were resuspended in the original volume of
42 156 PBS. The washing was repeated 5 times. The resulting
43 157 suspension was stored at + 4 °C. (The final suspension
44 158 concentration was controlled by weighing the compound
45 159 washed and precipitated five times from distilled water
46 159 and then fully dried.)

2.3. Immobilization of antibodies on the MNP

33 A solution of anti-AFB1 antibodies (2.8 mg/ml) was
36 163 added to 500 µl of a 3 mg/ml solution of MNP in PBS to a
37 164 final immunoglobulin content of 70 µg/ml. The solution
38 165 was incubated for 30 minutes with vigorous stirring by the
39 166 same way as described at Section 2.2. The particles were
40 167 precipitated by applying a magnetic field and supernatant

2. Experimental

2.1. Materials and Reagents
168 was separated with followed washing of the particles three 169 times with PBS. The resulting suspension was stored at + 4 170 °C.

171 The ratio of anti-AFB1 antibodies and MNP was chosen 172 based on our previous study 33 and accorded to the 173 monolayer immobilization. The immobilization yield of 174 antibodies (measured by ELISA based on the difference of 175 their content in the initial formulation and in the combined 176 supernatant) was equal to 24%.

177 2.4. Characterization of the MNP and its conjugate with 178 antibodies

179 During the transmission electron microscopy the 180 preparations of the MNP and its conjugate with antibodies 181 were applied to 300-mesh grids (Pelco International, USA, 182 www.pelcoint.com) coated with a support film of 183 poly(vinyl formal). The film was formed on a glass plate by 184 its incubation in formvar solution (0.15% in chloroform), 185 removed from the glass and used to cover the grids 34.

186 The images were obtained with a JEM CX-100 electron 187 microscope operating at 80 kV. The digital microscopic 188 images were analyzed with the Image Tool program 189 (University of Texas Health Science Center, San Antonio, 190 TX, USA).

191 Particle size determination was performed by dynamic 192 light scattering using a Zetasizer Nano. Before 193 measurement, the preparation was diluted by water to a 194 1:2000 ratio. Registration was carried out at 25 °C for 10 195 seconds at scattering angle 12.8°.

196 Zeta potential measurement was performed using a 197 Zetasizer Nano. Before measurement, the preparation was 198 diluted by water to a 1:100 ratio. Registration was carried 199 out at 25 °C at 2 mm measurement position.

200 2.5. Microplate ELISA for AFB1

201 Antibodies against AFB1 were incubated in a 202 microplate for 2 h at 37°C at a concentration of 1 µg/ml in 203 100 µl of PBS. After four washes with PBST, a solution of 204 AFB1 (50 µl) at concentrations between 3 ng/ml and 4 205 pg/ml in PBST were added, mixed with 50 µl AFB1-HRP 206 conjugate (100 ng/ml, by HRP) and incubated for 60 min at 207 37°C. The microplate wells were then washed four times 208 with PBST.

209 To determine the peroxidase activity, the substrate 210 solution (0.42 mM TMB and 1.8 mM H2O2 in a 0.1 M 211 sodium citrate buffer, pH 4.0; 100 µl per well) was 212 injected. After incubation at room temperature for 15 min, 213 the reaction was terminated by the addition of 100 µl of 214 M H2SO4. The absorbance of the reaction product was read 215 at 450 nm.

216 2.6. ELISA for AFB1 with the use of MNP

217 5 ml of AFB1 solutions at concentrations ranging from 218 1 ng/ml to 15 fg/ml was mixed with 50 µl of the MNP- 219 antibody conjugate at a concentration of 1 µg/ml (as MNP) 220 in PBST-BSA or in PBST-BSA containing either 5, 10, or 20% 221 methanol, or in corn extract containing 20% methanol and 222 incubated for 30 min at room temperature with vigorous 223 stirring. The MNP was precipitated by applying a magnetic 224 field; the supernatant was removed and 100 µl of PBST- 225 BSA was added (50-fold preconcentration) and the 226 precipitate was resuspended. The resulting concentrated 227 suspension was transferred to a microplate and washed 228 more time by precipitating in a magnetic field, removing 229 the supernatant, and resuspending in PBST-BSA. 100 µl of 230 AFB1-HRP conjugate was then added at a concentration of 231 600 ng/ml (in preliminary experiments, the concentration 232 was varied in the 200-600 ng/ml range). After 10 minutes 233 of incubation at room temperature with vigorous stirring, 234 the preparation was washed 4 times with PBST-BSA.

235 To determine the peroxidase activity the substrate 236 solution described above was added to the resulting 237 residue, then it was resuspended. Further manipulations 238 were performed similar to conventional microplate ELISA.

239 2.7. ELISA data processing

240 The plot of the absorbance (y) versus the antigen 241 concentration in the sample (x) was drawn with Origin 7.5 242 software (Origin Lab, Northampton, USA) using the four- 243 parameter function $y = (A-D)/(1+(x/c)^n) + D$. The analytical 244 characteristics of the assay, i.e. limit of detection and 245 working range, were determined based on the resulting 246 function, as described in 35, 36.

247 2.8. ELISA validation samples

248 The corn for the extract was bought at a store. Milled 249 grains were mixed with an extraction solution (70% 250 methanol, 30% water) at a ratio of 1:5, and incubated with 251 gentle stirring at room temperature for 1 day (in 252 accordance with Asis et. al. 37, with modifications). After 253 centrifugation, the supernatant was collected and stored 254 at 4°C. The extracts were analyzed by HPLC according to 255 Barbas et. al. 38 and no aflatoxin B1 was detected. Aflatoxin 256 B1 solution was introduced into the obtained extract, 257 immediately prior to performing the ELISA.

258 3. Results and discussion

259 3.1. Preparation and characterization of the reagents

260 When ELISA is carried out in the usual format, the 261 monoclonal antibodies against AFB1 used in this study 262 provided an AFB1 detection limit equal to 20 pg/ml.

263 Magnetite nanoparticles were obtained by the widely 264 used method based on co-precipitation of Fe2+ and Fe3+ 265 ions while treated with ammonia39. The co-precipitation 266 protocol has been selected on the basis of our previous 267 studies 33. The dimensional characteristics of the particles 268 in the resulting preparation were determined after 269 synthesis of MNP and their conjugation with antibodies by 270 means of transmission electron microscopy and optical 271 measurements of nanoparticles in bulk using a Zetasizer 272 Nano analyzer. Transmission microscopy shows that the 273 preparations of both MNP and their conjugates consist of 274 small particles that form the aggregates and chains (Fig. 1 275 (a) and (b)). By selecting images of individual MNP in the 276 micrographs, it can be seen that MNP average diameter 277 was 9.1±3.2 nm (see Fig. 1 (c)) and MNP conjugate average
The diameter of the MNP was 9.8±3.7 nm. The shape of the MNP was nearly spherical (axial ratio was 1.4). The aggregate dimensions varied significantly.

Fig. 1. TEM characterization of MNP and its conjugate. (a) – image of MNP; (b) – image of MNP conjugated with antibody; (c) – distribution of the nanoparticles (n = 116) by their average diameter measured by the TEM technique.

Enzyme immunoassay with magnetic preconcentration was performed according to the sequence of steps shown in Fig. 3. MNP conjugate with anti-AFB1 antibodies was added to a test sample potentially containing AFB1 (Fig. 3, a). After incubation, the resulting MNP-antibodies-antigen complex was separated from the free components by applying a magnetic field (Fig. 3, b); it was preconcentrated 50-fold by resuspension and transferred to a microplate well (Fig. 3, c). Next, peroxidase-labeled AFB1 was added to the microplate wells (Fig. 3, d). After incubation, washing and reacting with the substrate (Fig. 3, e), the analyte content was determined on the basis of the measured optical density. The higher the AFB1 content of the sample, the higher the binding of the AFB1-peroxidase conjugate with the substrate, and the analyte content was determined.

Thus, the proposed use of superparamagnetic nanoparticles (instead of the commonly used larger ones) in the analysis, on the one hand, ensured the maximum increase in the total surface area of nanoparticles for the immobilization of antibodies. On the other hand, the aggregation of superparamagnetic particles resulted in the emergence of larger agglomerates, which did not change their structure during several cycles of precipitation and resuspension. Thus, despite the small initial dimensions of the MNP, repeated separation in a magnetic field and washing of their agglomerates becomes possible during the ELISA, just like when working with traditionally used larger nanoparticles of magnetite.
optimal time of interaction between the magnetic immunosorbent and the AFB1-peroxidase conjugate was indicated to be 5 minutes.

**Fig. 5.** ELISA of AFB1 using MNP. Recorded signal (optical density) curves as functions of the AFB1 concentration, obtained at different interaction times of the magnetic immunosorbent with the AFB1-peroxidase conjugate: 1 – 5 min, 2 – 10 min, 3 – 60 min.

**Fig. 4.** ELISA of AFB1 using MNP. Recorded signal (optical density) curves as functions of the AFB1 concentration, obtained at 5-, 10- and 60-minute incubation were not significantly different in terms of the operating ranges of the content of AFB1, maximum sensitivity and low background noise (the signal due to non-specific sorption of the marker on the MNP surface).

**Fig. 3.** Proposed scheme of immunoenzyme assay using MNP: a-g - the sequence of steps (1 – antigen-containing sample; 2– MNP conjugate with specific antibodies; 3 – magnet; 4 – antigen-enzyme for conjugate; 5 – enzyme substrate).

The study of kinetics of the immunochemical reactions in the proposed system is of particular interest. Experimental results that vary the time for interaction between the MNP-antibodies conjugate and the free antigen and for interaction between the magnetic immunosorbent and the AFB1-peroxidase conjugate are shown in Fig. 4 and 5, respectively. As seen in Fig. 4, effective preconcentration of AFB1 from a large sample volume requires 30 minutes of incubation, which, by the way, is less than half of traditional ELISA. A shorter duration of this step only leads to partial binding of the antigen by the immunosorbent in the sample and, accordingly, to a shift in the ELISA calibration towards higher analyte concentrations. However, the increase of interaction time with the analyte to over 30 min does not significantly change the calibration function (see curves 2 and 3 in Fig. 4). Fig. 5 reflects the much more rapid interaction nature of the magnetic immunosorbent with the AFB1-peroxidase conjugate, when carried out in the small volume of microplate wells. The competing curves obtained at 5-, 10- and 60-minute incubation were not significantly different in terms of the operating ranges of detectable AFB1 concentrations. Moreover, a significant increase in incubation time causes unwanted nonspecific conjugate adsorption on the carrier, independent of the presence of antigen (curve 3 in Fig. 5). Accordingly, the optimal time of interaction between the magnetic pretreatment steps was 30 minutes.

A calibration curve for determining AFB1, shown in Fig. 6, was obtained under the selected ELISA conditions with the use of MNP. Analytical characteristics of this variant of ELISA, as well as those of traditional ELISA are given in Table 1.
this study provides the right combination of activities aimed at the speed and sensitivity of immunoassays.

Fig. 6. Calibration curve for AFB1 determination by the developed method.

Table 1. Comparison of analytical parameters of different ELISA versions for aflatoxin B1.

<table>
<thead>
<tr>
<th></th>
<th>Limit of detection, ng/mL</th>
<th>Working range, ng/mL</th>
<th>Assay duration, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNP-based ELISA with concentration, this study</td>
<td>0.002</td>
<td>0.002-0.2</td>
<td>50</td>
</tr>
<tr>
<td>Common format of ELISA, this study</td>
<td>0.015</td>
<td>0.015-0.5</td>
<td>120</td>
</tr>
<tr>
<td>MNP-based ELISA without concentration</td>
<td>0.02</td>
<td>0.02-1</td>
<td>20</td>
</tr>
</tbody>
</table>

3.3. Comparing the developed technique with other embodiments of magnetic preconcentration in ELISA

As noted above, in most of the studies on the application of MNP in ELISA the immunomagnetic sorbent is considered only as a means of separating the reactants but not as a means of analyte preconcentration from large samples volumes.

An exception is the work of Kuo et al. 19, who proposed a 1000-fold preconcentration of the analyte by using MNP, which lowered the detection limit 10-fold with a 2 hr. test duration. Preconcentration was also applied in the work of Kim et al. 18, who combined the use of MNP for extraction and preconcentration of the antigen and silicic nanoparticles with adsorbed fluorophore as a means of detection. A 54-fold reduction in enrofloxacin detection limit of was achieved through microscopy-aided recording of analytical results and prolonged incubation of the reagents; the total assay time was 3 hours.

The largest improvement in immunoassay, including analyte preconcentration by using MNP, is described in the work of Cho et al. 17. However, to achieve their goal, they used a cascade of three conjugates of antibodies with colloidal gold, two types of peroxidase-labeled antispecies antibodies, and analyte preconcentration using MNP. The total duration of the assay was 2.5 hours. Despite the record-lowering of the detection limit – by a factor of 10^5 – the methodology of the assay is very difficult, time-consuming, and characterized by accumulation of errors at each stage, as well as consumption of significant amounts of various reagents.

Thus, in the similar works by predecessors, the use of MNP provided only a lowering in analyte detection limits, but not a reduction in the duration of the ELISA process. Our proposed embodiment offers a gain both in sensitivity and in the duration of analysis (50 minutes in place of 120).

3.4. Validation of the developed ELISA

A specific feature of AFB1 as an antigen is its extremely low solubility in water. In this regard, organic solvents (mostly methanol) in high – 70% – concentrations are used for its extraction. Such high content of organics in the reaction mixture causes denaturation of proteins (including antibodies and enzymes), hindering the conduct of immunoassays. To prevent this effect, the test sample is diluted with aqueous saline solution before the analysis, resulting in an increase in the minimum concentration of detectable analyte in the sample, proportional to the degree of dilution. Therefore, while transitioning from pure model solutions of AFB1 to actual samples, the influence of the matrices on the assay sensitivity was estimated.

Experiments were performed wherein the reaction between the MNP-antibodies conjugate and the AFB1 containing sample took place in a medium with 20, 10, 5%, and trace amounts of methanol. It was found that the influence of the matrices on the assay sensitivity was 120% (see Table 2).

The observed stability of the magnetic immunosorbent to methanol (AFB1 extraction means from real samples) allows a minimization of the dilution of the test sample and, accordingly, results in a more sensitive analysis.

In view of this fact, the developed ELISA method was validated for AFB1 detection in corn plant extracts. The methanol content in the reaction mixture was 20%. ELISA was characterized by a range of detectable concentrations of AFB1 from 2 to 200 pg/ml, which corresponds to a mycotoxin content in the source grains preparation of 0.04 to 4.2 ng/g of grain. AFB1 recovery ranged from 74 to 120% (see Table 2).
Table 2. Recovery of aflatoxin B1 in the corn extract by applying the developed ELISA method with the use of magnetic nanoparticles.

<table>
<thead>
<tr>
<th>Introduced concentration of aflatoxin B1, pg/ml</th>
<th>Found concentration of aflatoxin B1, pg/ml</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>5.4</td>
<td>100</td>
</tr>
<tr>
<td>8.1</td>
<td>7.1</td>
<td>87.5</td>
</tr>
<tr>
<td>11.4</td>
<td>8.4</td>
<td>74.0</td>
</tr>
</tbody>
</table>

The proposed assay is an order of magnitude more sensitive (the detection limit was 20 pg/mL) as compared with the previously published one with the same reagents, but without pre-concentration.

It should be noted that the sensitivity of the immunochemical detection of AFB1, equal to 0.042 ng per 1 g of the tested plant sample is a good indicator. According to the COMMISSION REGULATION (EU) No 165/2010, the maximum allowable concentration of AFB1 in food is from 1 to 20 µg per kg, and for baby food it is reduced to 0.1 µg/kg. Consequently, the proposed ELISA method using MNP provides the practically demanded sensitivity of contaminant detection, allowing control of different types of foods and agricultural products.

Acknowledgments

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References
