1 Spectra analysis of coating antigen: a possible explanation for
2 difference in anti-AFB1 polyclonal antibody sensitivity

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20 **Abstract:**

21 For the detection of small hapten molecules, indirect competitive enzyme-linked
22 immunosorbent assay (icELISA) is a preferred method. However, diverse coating
23 antigen might bring different antiserum titer and sensitivity for the identical antiserum.
24 In the present study, four AFB1-protein (aflatoxin B1-carrier protein) conjugates were
25 prepared by activated ester method (AFB1O-BSA/AFB1O-OVA) and mannich method
26 (AFB1-cBSA/AFB1-cOVA), and then applied as coating antigen for titer and
27 sensitivity detection of the identical antiserum obtained from rabbit immunized by
28 AFB1-KLH. Afterwards, the ultraviolet-visible, fluorescence and far-ultraviolet
29 circular dichroism (far-UV CD) spectra were recorded for understanding the difference
30 in titer and sensitivity obtained. Results revealed that AFB1O-BSA/AFB1O-OVA
31 showed a strong intrinsic fluorescence band centered at 450 nm that originated from the
32 emission of AFB1, which differed from AFB1-cBSA/AFB1-cOVA, while the decrease
33 of α -helical and increase of β -sheet in AFB1-cBSA was the most remarkable. This
34 indicated that the better sensitivity obtained by using AFB1O-BSA as coating antigen
35 might be caused by its extended structure, because such structure affect the binding
36 between AFB1 and antibody. The study might offer structural information for
37 understanding the titer and sensitivity difference caused by coating antigen.

38
39 **Key words:** aflatoxin B1; fluorescence spectra; circular dichroism spectra;
40 heterogeneous ELISA.

41 **1 Introduction**

42 For the detection of small hapten molecules in enzyme-linked immunosorbent
43 assay (ELISA) systems, the hapten was usually cross-linked with some carrier proteins
44 to be used as immunogen or coating antigen. It was generally accepted that the design
45 of immunogen was crucial for high affinity antibody production, thus achieving
46 sensitive detection of hapten [1, 2], and a mountain of work was performed to
47 investigate the complete antigen preparation, such as the study of relationship between
48 epitope density [3, 4], modified hapten structure [5, 6] of immunogen and their
49 immunogenicity.

50 Actually, in addition to immunogen, hapten-carrier protein conjugate applied as
51 coating antigen also played an important role for sensitive detection of hapten in ELISA.
52 And one of the most well-known strategy was employing heterologous coating antigen
53 to improve detection sensitivity [7, 8]. However, how the sensitivity was affected by
54 coating antigen was poorly understood. As we all know, UV-Vis spectra, fluorescence
55 spectra and far-UV circular dichroism (CD) are excellent methods for rapidly
56 evaluating the secondary structure and properties of proteins [9, 10]. By means of
57 analysis with spectroscopic parameters, some clues to the difference of the coating
58 antigen can be obtained.

59 AFB1 is a toxic metabolite mainly produced by fungi of *Aspergillus flavus*, *A.*
60 *parasiticus* and *A. nomius* [11, 12] that contaminates a wide range of agricultural
61 products, therefore, it is of great significance to control AFB1 levels. To date, we have

performed a few researches on AFB1 [10, 13-15], including the preparation of antibodies and establishment of various immunoassays for rapid and sensitive detection. In the course of study, the observations that diverse coating antigen brought different sensitivity in ELISA prompted us to investigate the relationships between detection sensitivity and characteristics of coating antigen in detail. In the present study, four AFB1-protein (aflatoxin B1-carrier protein) conjugates were prepared by activated ester method (AFB1O-BSA/AFB1O-OVA) and mannich method (AFB1-cBSA/AFB1-cOVA), and then rabbit anti-AFB1 polyclonal antibody (Immunogen: AFB1-KLH) was employed for titer and sensitivity detection by using the four AFB1-protein conjugates as coating antigen. Subsequently, ultraviolet-visible, fluorescence and far-ultraviolet circular dichroism (far-UV CD) spectra were recorded for understanding the difference in titer and sensitivity obtained. The study performed would allow unique insights into the relationship between detection sensitivity and coating antigen.

2 Materials and methods

2.1 Materials

AFB1 was purchased from Alexis (Lausen, Switzerland). Bovine serum albumin (BSA), Ovalbumin (OVA), N-hydroxysuccinimide (NHS) and rabbit anti-AFB1 polyclonal antibody (Immunogen: AFB1-KLH) were obtained from Sigma (St. Louis, MO, USA). EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide) was purchased from Pierce (Rockford, IL, USA). Goat anti-rabbit IgG-HRP conjugate was purchased from Sino-American Biotechnology Company (Beijing, China). AFB1O was prepared according to the method of Chu et al. [16]. Cationic BSA (cBSA) and Cationic OVA

84 (cOVA) were prepared according to the method described by Zhou *et al.* [17]. All other
85 chemicals employed in the present study were either analytical pure or with higher
86 quality.

87 **2.2 Preparation of AFB1-protein conjugates**

88 **2.2.1 Preparation of AFB1O-BSA (OVA) by activated ester method**

89 For conjugation, 5 mg of BSA (OVA) was dissolved in 1 mL of 0.1 M NaHCO₃
90 solution (pH 8.3), and then aliquots of AFB1O solution (1 mg) activated by EDC and
91 (NHS) in dimethylformamide were added dropwise to the BSA (OVA) solution. The
92 mixture was agitated at room temperature for 16-20 h [18]. The conjugates of AFB1O-
93 BSA (OVA) were purified by ultrafiltration, and then stored at -20 °C.

94 **2.2.2 Preparation of AFB1-cBSA (cOVA) by Mannich method**

95 The preparation of AFB1-cBSA (cOVA) was performed as described by our
96 previous report [17].

97 **2.3 Determination of rabbit polyclonal antibody titer with different AFB1-protein** 98 **conjugates as coating antigen**

99 The determination of antibody titer with diverse coating antigen was conducted
100 through indirect non-competitive ELISA as described previously [19]. Briefly, 100 µL
101 of AFB1-protein conjugates (1.0 µg/mL) in bicarbonate buffer (pH 9.6) was added to
102 each plate well, and incubated at 4 °C overnight. Each well was washed three times
103 with PBST (0.1 M PBS, pH 7.4, containing 0.05% Tween 20), and decanted.

104 Subsequently, 5% skim milk in PBST was added, and incubated for 2 h at 37 °C to
105 eliminate non-specific binding. After the same washing procedure, 100 µL of serially
106 double diluted anti-AFB1 serum was added, and incubated at 37 °C for 1 h. After three
107 times washing, another 1 h incubation was conducted with 100 µL of goat anti-rabbit
108 IgG-HRP conjugate (1/4000) in PBST. And then, 100 µL of peroxidase substrate (40
109 mg of 1,2-diamino-benzene and 150 µL H₂O₂ in 100 mL of citrate buffer, pH 4.5) was
110 added after five times washing, and incubated for 15 min at 37°C. The reaction was
111 terminated by adding 50 µL of 2 M sulfuric acid. Absorbance at 492 nm was determined
112 in a Multiskan MK3 ELISA reader (Thermo, China). The antibody titer was expressed
113 as the dilution factor of antibody that produced an OD_{492 nm} value of about 1.5. Wells
114 coated with carrier proteins was used as control.

115 **2.4 Determination of antibody sensitivity in serum with different AFB1-protein** 116 **conjugates as coating antigen**

117 Indirect competitive ELISA (icELISA) was employed for sensitivity detection,
118 and the procedure was similar to that mentioned in Section 2.3 except replacement of
119 100 µL of double diluted anti-AFB1 serum with 90 µL of suitable diluted anti-AFB1
120 serum and 10 µL of different concentrations (0.1, 1, 5, 10, 100, 1000 ng/mL) of AFB1
121 in methanol solution.

122 The IC₅₀ value, the value of 50% binding inhibition, represented the sensitivity of
123 anti-AFB1 antisera.

124 **2.5 Characterization of AFB1-protein conjugates**

125 **2.5.1 UV-Vis analysis**

126 The absorption spectra of AFB1-protein conjugates in water were scanned from
127 200 nm to 500 nm at 1.0 nm intervals, employing a double bundled UV-Vis
128 spectrometer, model UV-1700 (Shimadzu, Japan) equipped with quartz cuvettes with a
129 path-length of 10 mm. The conjugate molar ratio of AFB1 to each carrier protein was
130 calculated according to the method described by Zhou *et al.* [17]. The scanning speed
131 was 1500 nm/min.

132 **2.5.2 Fluorescence spectra analysis**

133 The fluorescence spectrum was recorded at room temperature on a RF-5301PC
134 model spectrofluorimeter (Shimadzu, Japan) equipped with a Xenon lamp source.
135 Fluorescence spectrum of AFB1, different carrier proteins and AFB1-protein
136 conjugates all measured at excitation wavelength 280 nm and 363 nm, and the
137 corresponding grating slit 3/3 nm.

138 **2.5.3 Far-UV CD spectra analysis**

139 The far-UV CD measurement was performed on a JASCOJ-810S circular
140 dichroism spectropolarimeter (Japan Spectroscopic, Japan) using a 0.1 cm cell at 0.1
141 nm intervals, with three scans averaged for each far-UV CD spectrum, the bandwidth
142 was 1.0 nm and the scanning speed was 200 nm/min.

143 **3 Results**

144 **3.1 Determination of antibody titer with different AFB1-protein conjugates**

145 The titers of rabbit anti-AFB1 polyclonal antibody determined by using different
 146 AFB1-protein conjugates as coating antigen were shown in Fig. 1. The results
 147 demonstrated that rabbit anti-AFB1 polyclonal antibody exhibited different titer when
 148 employing diverse coating antigen, and wells immobilized with AFB1O-BSA/AFB1O-
 149 OVA brought about a higher titer than those immobilized with AFB1-cBSA/AFB1-
 150 cOVA. In order to check if the higher titer was caused by non-specific binding of carrier
 151 protein, the antibody titer was also evaluated with carrier proteins (BSA, cBSA, OVA,
 152 cOVA) as coating antigen. According to the results, the cross-reaction between each
 153 carrier protein and antibody was weak, and the non-diluted antibody did not cause a
 154 value in OD_{492nm} larger than 0.5, indicating that all the non-specific binding of carrier
 155 proteins were not strong enough to cause the titer difference.

156
 157 **Fig. 1** The titer curves of rabbit anti-AFB1 polyclonal antibody using different AFB1-protein conjugates
 158 as coating antigen.

159 3.2 Determination of antibody sensitivity with different AFB1-protein conjugates 160 by indirect competitive ELISA

161 According to the results of antibody titer determination, the corresponding

162 concentration of antibody was used in each sensitivity measurement. As described in
 163 **Section 2.4**, the competitive inhibition curve of rabbit anti-AFB1 polyclonal antibody
 164 using diverse coating antigen obtained through icELISA was shown in Fig. 2. And
 165 Table 1 illustrated detection sensitivity and linear equation of antibody with different
 166 AFB1-protein conjugates as coating antigen. As can be seen, the sensitivity of rabbit
 167 polyclonal antibody obtained with diverse AFB1-protein conjugates as coating antigen
 168 was different; however, when the concentration of AFB1 was in the range of 0.1-10
 169 ng/mL or 1-10 ng/mL, good correlations were presented between the concentration of
 170 AFB1 and competitive inhibition rate. Notably, a better sensitivity was obtained when
 171 applying AFB1O-BSA (OVA) as coating antigen, which indicated that the homologous
 172 system yielded a little more sensitive assay, and this was consistent with previous report
 173 [20]. AFB1-cOVA/AFB1-cBSA conjugates resulted in the least amount of inhibition of
 174 antibody binding, which indicated that the ability of AFB1O-BSA/AFB1O-OVA to
 175 specifically bind with anti-AFB1 serum was different from AFB1-cOVA/ AFB1-cBSA.

176
 177 **Fig. 2** The indirect competitive ELISA inhibitory curves of rabbit anti-AFB1 polyclonal antibody with
 178 different AFB1-protein conjugates as coating antigen

179 3.3 UV-Vis analysis

As can be seen from Fig. 3, all the AFB1-protein conjugates exhibited broad characteristic absorption peaks at 270 nm and 363 nm. The band around 270 nm indicated a combination band of 262 nm and 280 nm due to AFB1 and carrier protein, respectively. Judging by the presence of absorption peak of AFB1 at 363 nm, each AFB1-protein was well conjugated.

Fig. 3 The UV-Vis spectra of 0.51 mg/mL AFB1-cBSA, 0.50 mg/mL AFB1O-BSA (A), 0.66 mg/mL AFB1-cOVA and 0.56 mg/mL AFB1O-OVA (B).

One of the important parameters of conjugates is the molar ratio between hapten and carrier protein. The molar ratio plays an important role, with regard to the degree of immune reactivity, solubility of the final conjugate, and the sensitivity of the immunoassay based on such antibodies elicited by the corresponding conjugate [21]. The molar ratio of $n_{\text{AFB1O}} : n_{\text{BSA}}$, $n_{\text{AFB1O}} : n_{\text{OVA}}$, $n_{\text{AFB1}} : n_{\text{cBSA}}$ and $n_{\text{AFB1}} : n_{\text{cOVA}}$ were 3.53:1, 3.21:1, 4.42:1, 3.97:1, respectively. Notably, there was slight difference in absorption profile and molar ratio among four AFB1-protein conjugates.

3.4 Fluorescence spectra analysis

196 Spectroscopic tools often deliver structural information on binding-induced
197 conformational changes of carrier protein and hapten [22]. Fluorescence spectroscopy
198 has been particularly useful in the area of ligand binding, and thus steady state
199 fluorescence quenching is the primary techniques under discussion.

200 BSA and OVA have three intrinsic fluorophores: tryptophan, tyrosine and
201 phenylalanine that can quench. In fact, because phenylalanine has a very low quantum
202 yield and the fluorescence of tyrosine is almost totally quenched if it is ionized or near
203 an amino group, a carboxyl group or a tryptophan, the intrinsic fluorescence of BSA
204 and OVA is mostly due to the tryptophan residues [23].

205 From the regular fluorescence spectra (Fig. 4), valuable information on the binding
206 of carrier protein to AFB1 can be obtained. As can be seen in Fig. 4(A) and (C), BSA
207 (OVA) and cBSA (cOVA) aqueous solution exhibited a strong intrinsic fluorescence
208 band centered at about 340 nm originated from the emission of tryptophan residues,
209 whereas AFB1 had a strong quenching effect on carrier protein at 340 nm in AFB1-
210 protein conjugates. However, the AFB1O-BSA/AFB1O-OVA showed a strong
211 intrinsic fluorescence band centered at 450 nm originated from the emission of AFB1,
212 which differed from AFB1-cBSA/AFB1-cOVA.

Fig. 4 The regular fluorescence spectra of AFB1O, AFB1-protein conjugates and carrier proteins (distilled water, pH 7.0)

Synchronous fluorescence spectra can provide information about the molecular environment, particularly in the vicinity of fluorophore functional groups. When the $\Delta\lambda$ ($\Delta\lambda = \lambda_{\text{emi}} - \lambda_{\text{exc}}$) was set as 15 and 60 nm, the synchronous fluorescence gave characteristic information for the tyrosine residues and tryptophan residues. For the synchronous fluorescence spectra of carrier proteins and AFB1-protein conjugates (Fig. 5), AFB1 had a quenching effect on tyrosine residues and tryptophan residues, and the fluorescence peaks of tyrosine residues and tryptophan residues both had a slight blue shift in AFB1-protein conjugates, indicating its polarity was decreased, and the hydrophobicity was increased after coupling [24]. However, there was no obvious difference among the four AFB1-protein conjugates.

Fig. 5 Synchronous fluorescence spectra of AFB1-protein conjugates and carrier proteins

3.5 Far-UV CD spectra analysis

CD is a spectroscopic technique based on the difference in absorption of left and right circularly polarized light. Far UV CD spectroscopy was usually applied to determine its effectiveness in assessing the secondary structure of protein [25]. The far-UV CD spectra of different proteins at room temperature showed in Fig. 6. The calculation of secondary structure was conducted using Dichroweb (<http://dichroweb.cryst.bbk.ac.uk/html/home.shtml>) with CDSSTR references set optimized for wavelengths from 190 nm to 260 nm [26], and the results were shown in Table 2. The far-UV CD spectra of BSA (OVA) exhibited two negative bands in the ultraviolet region at 208 and 222 nm, which indicated that the structure of BSA (OVA) was predominantly α -helical [27]. However, the negative peaks intensity was significantly reduced in AFB1-cBSA/AFB1-cOVA compared to AFB1O-BSA/AFB1O-OVA. The CDSSTR software analysis showed that the α -helical content of AFB1-cBSA decreased while the β -sheet content increased, indicating a more extended structure, which might affect the binding of AFB1 and antibody. According to findings of Goodrow *et al* (1998) [28], a lower affinity between coating antigen and antibody brought better detection

244 sensitivity in icELISA when the affinity of target and antibody was constant. And this might
 245 be the one of the reasons for explaining different sensitivity.

247 **Fig. 6** The far-UV CD spectra of AFB1-protein conjugates and carrier proteins

248 **4 Discussion**

249 ELISA performed in this study revealed immunoreactivity of all AFB1-protein
 250 conjugates. However, wells coated with AFB1O-BSA/AFB1O-OVA exhibited a higher
 251 titer than those immobilized with AFB1-cBSA/AFB1-cOVA. And Fig. 2 showed little
 252 antibody cross-reactivity of BSA (OVA), in comparison with cBSA (cOVA). In
 253 addition, binding of antiserum to AFB1-protein conjugates prepared by the activated
 254 ester method was always better inhibited by free hapten as compared to conjugates
 255 prepared by the Mannich method. All these results suggested that the weak inhibition
 256 observed with AFB1-cBSA and AFB1-cOVA might be related to methods for
 257 conjugating [29].

258 In the present study, the sensitivity of anti-AFB1 antiserum determined with
 259 diverse AFB1-protein conjugates were different. The reasons might be as follows: (A)
 260 although the methods of preparation for AFB1O-BSA/AFB1O-OVA and AFB1-
 261 cBSA/AFB1-cOVA were different, the results of IC_{50} were similar, suggesting that the

262 dissimilarity in their structure was probably not distinct. Thus, there was no obvious
263 difference in the sensitivity of antiserum [17]. (B) The AFB1-protein conjugates might
264 have different effective epitope densities, although the AFB1-protein molar ratios used
265 in the procedure for their preparation and apparent epitope densities detected by UV-
266 Vis were similar in all cases [18]. (C) The more extended structure of AFB1-
267 cBSA/AFB1-cOVA [17]. On the other hand, the differences in detection sensitivities
268 was not that large, implying that the antibody [has a high affinity for each conjugate](#),
269 and this was consistent with report of Burn *et al* (2004) [30], in which, they found high
270 affinity of antibody hardly enhanced detection sensitivity when employing
271 heterogeneous ELISA.

272 UV-Vis, fluorescence and far-UV CD spectroscopy were applied to analyze AFB1-
273 protein conjugates. UV-Vis results proved that carrier protein conjugated with AFB1
274 indeed exhibited similar curves. Fluorescence analysis results proved that the AFB1O-
275 BSA/AFB1O-OVA showed a strong intrinsic fluorescence band centered at 450 nm that
276 originated from the emission of AFB1, which differed from AFB1-cBSA/AFB1-cOVA.
277 The CDSSTR software analysis suggested that the α -helical content of AFB1-cBSA
278 increased, while the β -sheet content decreased, which suggested that peptide chain
279 contracted and protein hydrophobicity increased. The significant differences of
280 fluorescence and far-UV CD spectroscopy between AFB1O-BSA/AFB1O-OVA and
281 AFB1-cBSA/AFB1-cOVA might be one of the evidences that explain the different
282 ability of AFB1-protein conjugates to bind anti-AFB1 serum.

283 In conclusion, different AFB1-protein conjugates used as coating antigen showed

different titer and sensitivity of the identical anti-AFB1 serum, and the significant differences in fluorescence and far-UV CD spectroscopy might be one of the evidences of difference. The study might provide useful information for understanding the titer and sensitivity difference caused by coating antigen.

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341

342 **Figure captions**

343 **Fig. 1** The titer curves of rabbit anti-AFB1 polyclonal antibody using different AFB1-
344 protein conjugates as coating antigen.

345 **Fig. 2** The indirect competitive ELISA inhibitory curves of rabbit anti-AFB1 polyclonal
346 antibody with different AFB1-protein conjugates as coating antigen

347 **Fig. 3** The UV-Vis spectra of 0.51 mg/mL AFB1-cBSA, 0.50 mg/mL AFB1O-BSA (A),
348 0.66 mg/mL AFB1-cOVA and 0.56 mg/mL AFB1O-OVA (B).

349 **Fig. 4** The regular fluorescence spectra of AFB1O, AFB1-protein conjugates and
350 carrier proteins (distilled water, pH 7.0)

351 *The proteins and AFB1O concentrations were 0.2 mg/mL and 30 ng/mL,

352 respectively. The excitation wavelength was 280 nm (A), (C) and 362 nm (B), (D).

353 **Fig. 5** Synchronous fluorescence spectra of AFB₁-protein conjugates and carrier
354 proteins

355 *The concentration of proteins was 0.2 mg/mL in distilled water. The solid and
356 hollow tags represented the synchronous fluorescence of tryptophan and tyrosine
357 residues, respectively.

358 **Fig. 6** The far-UV CD spectra of AFB₁-protein conjugates and carrier proteins

359 *The concentration of proteins was all 0.25 mg/mL in distilled water.

360

361 **Table captions**

362 **Table 1** Related parameters of indirect competitive ELISA inhibitory curves of rabbit
363 anti-AFB₁ polyclonal antibody with different AFB₁-protein conjugates as coating
364 antigen

365 **Table 2** Fractions of different secondary structures of proteins determined by CDSSTR

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371 **Table 1** Related parameters of indirect competitive ELISA inhibitory curves of rabbit anti-AFB1
 372 polyclonal antibody with different AFB1-protein conjugates as coating antigen

Coating antigen	linear ranges (ng/mL)	sensitivities (ng/mL)	linear equation
AFB1O-BSA	0.1-10	1.44	$y = -41.419x + 56.616, R^2 = 0.9924$
AFB1-cBSA	1-10	2.57	$y = -53.553x + 71.918, R^2 = 0.9862$
AFB1O-OVA	0.1-10	1.68	$y = -41.06x + 59.3, R^2 = 0.9867$
AFB1-cOVA	1-10	4.20	$y = -52.654x + 82.798, R^2 = 0.9772$

373

374 **Table 2** Fractions of different secondary structures of proteins determined by CDSSTR

	α -helix	β -sheet	Turns	Unordered	Total
BSA	0.45	0.14	0.16	0.26	1.01
cBSA	0.13	0.29	0.23	0.33	0.98
AFB1O-BSA	0.36	0.20	0.21	0.24	1.01
AFB1-cBSA	0.18	0.27	0.24	0.31	1
OVA	0.13	0.34	0.23	0.3	1
cOVA	0.10	0.32	0.23	0.32	0.97
AFB1O-OVA	0.13	0.31	0.23	0.31	0.98
AFB1-cOVA	0.08	0.34	0.23	0.33	0.98

375

Sensitivity detection

**different
coating
anigen**

Spectral characterization