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Kinetic insight into the mechanism of cholinesterasterase inhibition by aflatoxin B1 to develop biosensors

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ABSTRACT

In this paper, the inhibition effect of aflatoxin B1 on different species of cholinesterases was investigated to unravel action mechanism. The inhibition curves of several cholinesterase mutants (obtained by spectrophotometric measurements of enzyme activity, pS curves) were analyzed. They showed that this toxin reversibly inhibits cholinesterases by binding to a peripheral site located at the entrance of the active site gorge without entering inside the site. Electric eel enzyme revealed the highest inhibition extent with a binding constant estimated to 0.35 μM. This binding prevents the entrance of substrate en route to the catalytic site and also decreases chemical steps of the reaction at the catalytic site: acetylation is reduced to the half and deacetylation is reduced to the third. Electric eel acetylcholinesterase was used to settle an amperometric biosensor. The best detection was obtained by using 0.3 mU enzyme on the electrode and 0.5 mM ATCh in the solution. The limit of detection was 3 μM corresponding to 20% inhibition.

1. Introduction

Mycotoxins are fungal secondary products commonly present in contaminated food or animal feeds that can cause severe illnesses. The optimal growth temperature of moulds with mycotoxin production ranges between 24 and 35 °C and thus, affection of crops is mainly concerns humid and warm zones, principally subtropical and tropical countries (Williams et al., 2004) with important losses in terms of human health, animal health, and condemned agricultural products (Shane, 1994). Factors contributing to the presence or production of mycotoxins in food or feed include storage and environmental or ecological conditions. Aflatoxins, an especially aflatoxin B1 (AFB1), are one of the most toxic groups of mycotoxins. AFB1 is principally produced by *Aspergillus flavus* and *Aspergillus parasiticus* which afflict amylaceous semen like grains, maize, peanuts and pistachios. Aflatoxins structure was first time described in 1965 (Asao et al., 1965). Acute intoxication manifests as hepatotoxicity (Cullen and Newberne, 1994). Last severe outbreak of aflatoxicosis in humans took place between 2004 and 2005 in Kenya with over 150 deaths (Azziz-Baumgartner et al., 2005). Chronic subsymptomatic exposure provokes hepatic damage as well as nutritional and immunosuppressive effects like anorexia and decreased T-cell function (Williams et al., 2004; Azziz-Baumgartner et al., 2005; Hussein and Brasel, 2001). The known LD_{50} values for different animal species range between 0.3 and 10 mg/kg bodyweight (Wogan, 1966) and estimation of LD_{50} value for humans lies between 2 and 6 mg/kg bodyweight. Thus, a man with a weight of 70 kg would therefore die from an approximate single ingested dose of 140–420 mg with a probability of 50%.

Besides acute and chronic symptoms of aflatoxicosis, bioactivation of aflatoxin B1 by cytochrome P450 to its highly reactive aflatoxin-8-9-epoxide results in DNA adducts which induce mutagenic and carcinogenic effects in a dose-dependent manner. Most commonly aflatoxin B1 causes liver cancer especially in combination with other hepatic risk factors like hepatitis B virus infection (Croy et al., 1978; Muench et al., 1983; Bennett et al., 1981; Foster et al., 1983; Vinitketkumnuen et al., 1997). Consumption over a long time increases the risk of developing a hepatocarcinoma due to the accumulation of aflatoxin B1 in the liver during that life span. Thus, the WHO-International Agency for Research on Cancer described naturally occurring mixtures of aflatoxins in the environment to be

Abbreviations: AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; ATCh, acetylthiocholine.

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cancerogenic to humans and aflatoxin B1 was placed as a Class 1 carcinogen.

Due to its high toxicity, the U.S. Food and Drug Administration created a standard regulating the aflatoxins amount in food. It set the maximal value of overall aflatoxin B1 affliction at 20 ppb in grains and at 0.5 ppb in milk (U.S. Food and Drug Administration, 1995. Sec. 555.400) and European Union set limits of total aflatoxin concentration in groundnuts and cereals intended for direct human consumption to 4 ppb (commission regulation no 466/2001 of 8 March 2001).

In order to monitor and to regulate the food trade market in accordance to the established standards, routine analysis systems are required. Currently, qualitative and fast analysis of aflatoxin B1 traces is commonly carried out by ELISA immunoassays (Micheli et al., 2005; Piermarini et al., 2007; Zollner and Mayer-Helm, 2006). On the other hand, quantification is usually made using gas chromatography–mass spectrometry, thin-layer chromatography, liquid chromatography–mass spectrometry or high-performance liquid chromatography after extraction of aflatoxin B1 from contaminated food samples (Zollner and Mayer-Helm, 2006; Sugita-Konishi et al., 2006). However, sample analysis by these conventional methods is quite expensive and time consuming.

Recent reports suggest that biosensors using cholinesterase as biological component may be used to detect aflatoxin B1: aflatoxicosis has been correlated to a diminution of acetylcholine turnover in rat brain (Egbunike and Ikegwuonu, 1984), aflatoxin B1 inhibits acetylcholinesterase with a non-competitive inhibition pattern (Cometa et al., 2005) and further, a colorimetric biosensor using electric eel acetylcholinesterase was recently described (Arduini et al., 2007). The attempt of our study was to understand more about the mode of aflatoxin B1 action. We first wanted to establish if acetylcholinesterase (AChE) is inhibited by aflatoxin B1 at the same concentration range as it is known to induce acute toxic symptoms to humans. The second objective was to unravel the mechanism of AChE inhibition by steady state kinetic measurements and by using *in vitro* mutagenesis. The third attempt was to develop a field-compatible biosensor and to determine the detection limit.

2. Experimental procedures

2.1. Materials

Drosophila melanogaster AChE (Dm-AChE) were produced in baculovirus system and purified as already described (Chaabihi et al., 1994). Mutants were obtained as previously described (Boublik et al., 2002). Human AChE (Hu-AChE), human BuChE (Hu-BuChE) and *Electrophorus electricus* AChE (Ee-AChE) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *Torpedo californica* AChE (Tc-AChE) was kindly supplied for Lilly Toker and Israel Silman. Aflatoxin B1 (MW: 312) and acetylthiocholine (MW: 289) were from Sigma. Aflatoxin B1 was dissolved in ethanol (1 mM) and subsequent dilutions were made in water.

2.2. Kinetics

Hydrolysis of ATCh was measured using the Ellman method (Ellman et al., 1961). Briefly, the activity of the enzyme at 25 °C was evaluated by following the absorbance at 412 nm in 25 mM phosphate buffer, pH 7 for 1 min after the addition of the substrate to the mixture containing buffer, Ellman reagent, enzyme and AFB1. Chemical hydrolysis of substrate was subtracted. Substrate concentrations range was 10 μM to 300 mM, with a minimum of two repetitions per concentration value. The tested AFB1 concentrations were 1, 10, 50 and/or 100 μM, depending on the reaction. Data were analysed using the model and equation of Stojan et al. (2004a,b) for ATCh hydrolysis inhibition. Fittings were performed by multiple non-linear regressions using the program GOSA-fit (www.bio-log.biz).

2.3. Immobilization of Ee-AChE on electrodes and biosensor measurements

The electrodes were modified with cobalt-phthalocyanine as mediator (Gwent Electronic Materials, UK). 3 μL of a 1:1 mixture of polyvinylalcohol and the enzyme solution were automatically deposited on the working electrode surface with a micropipette and polymerized under neon light at 4 °C for 3 h. Sensors were then left in the dark at 4 °C for at least further 24 h.

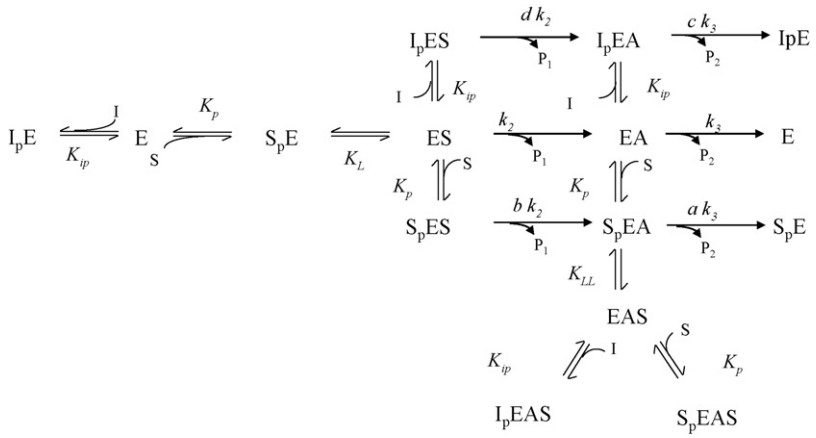
The electrodes were then measured using a potentiostat in 10 ml of 100 mM phosphate buffer, pH 7 at 30 °C. ATCh was added to the buffer solution and it was hydrolysed by the immobilized enzymes. The mediator cobalt-phthalocyanine oxidised the product of the ATCh hydrolysis (thiocholine) and it was thus, reduced. The reoxidation of the mediator in the working electrode at +100 mV vs. Ag/AgCl pseudo-reference electrode generated a current that was linked to a flatbed recorder. In this work, the stability of the electrodes was checked by adding consecutively a fixed amount of substrate several times. Only when the current was the same after at least three measurements, the electrodes were considered stable and were incubated in phosphate buffer containing AFB1. The decrease in the substrate steady state current after addition of AFB1 was used to evaluate the inhibition extent. All measurements were accomplished with a minimum of two repetitions.

3. Results

3.1. Inhibition type and binding site

Regarding the literature, the kinetics of inhibition were analyzed using Dm-AChE because the pS curve displays activation and inhibition by substrate, allowing the estimation of some parameters describing substrate binding and hydrolysis (Stojan et al., 2004a,b; Colletier et al., 2006). It has been found that AFB1 inhibited cholinesterase activity in the μM range. However, no effect could be reported on the enzyme activity when pre-incubating acetylcholinesterase with AFB1 since it remained constant along the measurement time (5 min after addition of the last reaction component). Thus, AFB1 reversibly inhibits Dm-AChE, as it has been recently reported for Ee-AChE (Arduini et al., 2007). According to this result, the steady-state inhibition kinetics of AFB1 was analyzed by mixing enzyme, substrate and AFB1 and measuring the activity for 1 min.

The AFB1 binding model was analyzed using the model for acetylthiocholine (ATCh) hydrolysis with inhibitor involvement (Scheme 1). In this model, the substrate initially binds to the peripheral site (PAS), mainly consisting of Trp321 (Dm-AChE numbering), at the entrance of the active-site gorge, thereby resulting in the complex SpE. Gliding down the gorge to the catalytic site results in the formation of a Michaelis addition complex (ES), thus, setting free the peripheral site for another substrate molecule to give a ternary complex (SpES). At this point, the enzyme is acetylated (acetylated enzyme, EA, and SpEA) and choline is then released. In the next step, a water molecule approaches to the esteratic bond and induces deacetylation to regenerate the free enzyme (E). However, the binding of another substrate molecule to the peripheral site of the Michaelis complex, leading to SpES, may block the choline exit. Substrate binding at the peripheral site of the acetylated enzyme usually increases deacetylation ($a > 1$). However, the substrate molecule bound to the peripheral site can also enter deeper



Scheme 1. Kinetic scheme used to analyse aflatoxin inhibition. 'E' represents the free AChE, 'EA' the acetylated enzyme, 'S' the substrate and 'I' the inhibitor. When a ligand is bound to the peripheral site, it is written on the left of 'E' with the subscript "p" added (SpE). When a ligand is bound at the catalytic site, the symbol is written on the right of 'E' (ES). Thus, SpEAS means that a substrate molecule is at the peripheral site and another substrate molecule at the catalytic site of acetylated enzyme. The general scheme for inhibitors was reduced for AFB1 by discarding the parts of the inhibitor binding at the catalytic site.

in the gorge of the acetylated enzyme (K_{LL}) thus, giving the EAS complex. At this stage, no water molecule can approach the ester bond and, as a consequence, the substrate molecule completely blocks deacetylation. Finally, if another substrate molecule binds to the peripheral site, a ternary complex between acetylated enzyme and two substrate molecules is formed (SpEAS). The derived initial rate equation for this model has been described in Stojan et al. (2004b).

Reversible inhibitors of AChE bind on the peripheral anionic site (PAS) and/or on the catalytic anionic site (CAS). Both binding positions prevents substrate hydrolysis either by corking the entrance to the active site (binding to PAS) or by the occupation of the choline binding site at the bottom of the gorge. The complete scheme corresponding to the binding to these two sites and its corresponding initial rate equation has been deeply detailed in Fekonja et al. (2007). Both binding sites can be distinguished with the fitting of the data by considering the presence of each complex: when the complexes IpE, IpES and IpEA are required for the fitting, the inhibitor binds to the peripheral site, but when the complexes EI, SEI are necessary, the inhibitor binds to the catalytic site. Whether all complexes are necessary, the inhibitor binds to both sites. The simplest model that fits on AFB1 inhibition data of substrate hydrolysis catalyzed by Dm-AChE was described as the binding of AFB1 to the peripheral site (Fig. 1A, Scheme 1, Eq. (1)) with an affinity estimated to 2.39 μM. Using an equation for the model of AFB1 binding in the catalytic site did not improve the fitting, showing that inhibition only results from the binding to the entrance of the active site, i.e. at the PAS. This binding impeded the entrance of substrate to the catalytic site, reduced deacetylation and increased the hindrance of choline exit thus, inhibiting the substrate hydrolysis. As substrate binds on the PAS before entering into the catalytic site, AFB1 behaved as a competitive inhibitor.

$$\frac{v}{[Et]} = \frac{k_3 \cdot k_2 \cdot [S]}{\frac{\left(k_3 \cdot K_p \cdot K_L \cdot \frac{1+[S]}{K_p} + \frac{[S]}{K_p \cdot K_L} + \frac{[S]^2}{K_p{}^2 \cdot K_L} + \frac{[S] \cdot [I]}{K_p \cdot K_{ip} \cdot K_L} + \frac{[I]}{K_{ip}}\right)}{\left(1 + \frac{b \cdot [S]}{K_p} + \frac{d \cdot [I]}{K_{ip}}\right)} + \frac{[S] \cdot k_2 \cdot \left(1 + \left(\frac{[S]}{K_p} + \frac{[S]}{K_p \cdot K_{LL}} + \frac{[S]^2}{K_p{}^2 \cdot K_{LL}} + \frac{[S]}{K_p} \cdot \frac{[I]}{K_{ip} \cdot K_{LL}} + \frac{[I]}{K_{ip}}\right)\right)}{1 + a \cdot \frac{[S]}{K_p} + c \cdot \frac{[I]}{K_{ip}}}} \quad (1)$$

Trp321, located at the entrance of the active site gorge is an essential residue of the PAS of Dm-AChE (corresponds to Trp279 in the Tc-AChE used for structural analysis). The binding of AFB1 to this site was verified by mutating this amino acid to alanine and the inhibition of the mutant by AFB1 was examined (Fig. 1B). Below 10 μM of AFB1 no inhibition could be observed, confirming that the binding of aflatoxin on the peripheral site was responsible for the inhibition observed with the wild type.

3.2. Sensitivity of cholinesterases to aflatoxin B1

Four species of AChE (beside Dm-AChE) were examined to find an acetylcholinesterase enzyme that reveals a high sensitivity towards AFB1. At AFB1 concentration ranging from 1 to 10 μM, inhibition was observed for all tested species except for Hu-BuChE (Fig. 1C–F). Hu-BuChE did not have a tryptophan in the PAS site, which was consistent with the lack of inhibition obtained even at 100 μM AFB1.

The affinity constants for the binding of AFB1 at the peripheral site (K_{ip} values) for Hu-AChE and Tc-AChE were in the same range than those obtained for Dm-AChE (~5 μM, Table 1). The lowest K_{ip} for the three inhibited enzyme species corresponded to Ee-AChE (0.5 μM). The effect of AFB1 on several *Drosophila* enzymes with mutations located at the entrance of the active site (R70V, Y71A, Y71D, Y71K, Y73A, W321L, W321N, W321del, L328F, Y370A) by fast screening of the hydrolysis of 1 mM ATCh at aflatoxin B1 concentrations of 1 and 10 μM was analyzed. However, anyone of these AChEs did not show more sensitive than Ee-AChE to AFB1.

3.3. Determination of the lowest aflatoxin B1 concentration detectable using an amperometric biosensor

Among all of examined AChEs, Ee-AChE was found to be inhibited by the lowest AFB1 concentrations and was used for developing the enzymatic biosensor. Initially, the optimal substrate concentration was established. The maximum inhibition was observed between 200 μM and 1 mM ATCh. At higher concentrations, reference and aflatoxin B1 curves converge as expected for a competitive inhibitor and further, substrate inhibition appears. Hence, no inhibition by the toxin could be observed at high substrate concentrations (Fig. 1). On the other hand, below 10 μM

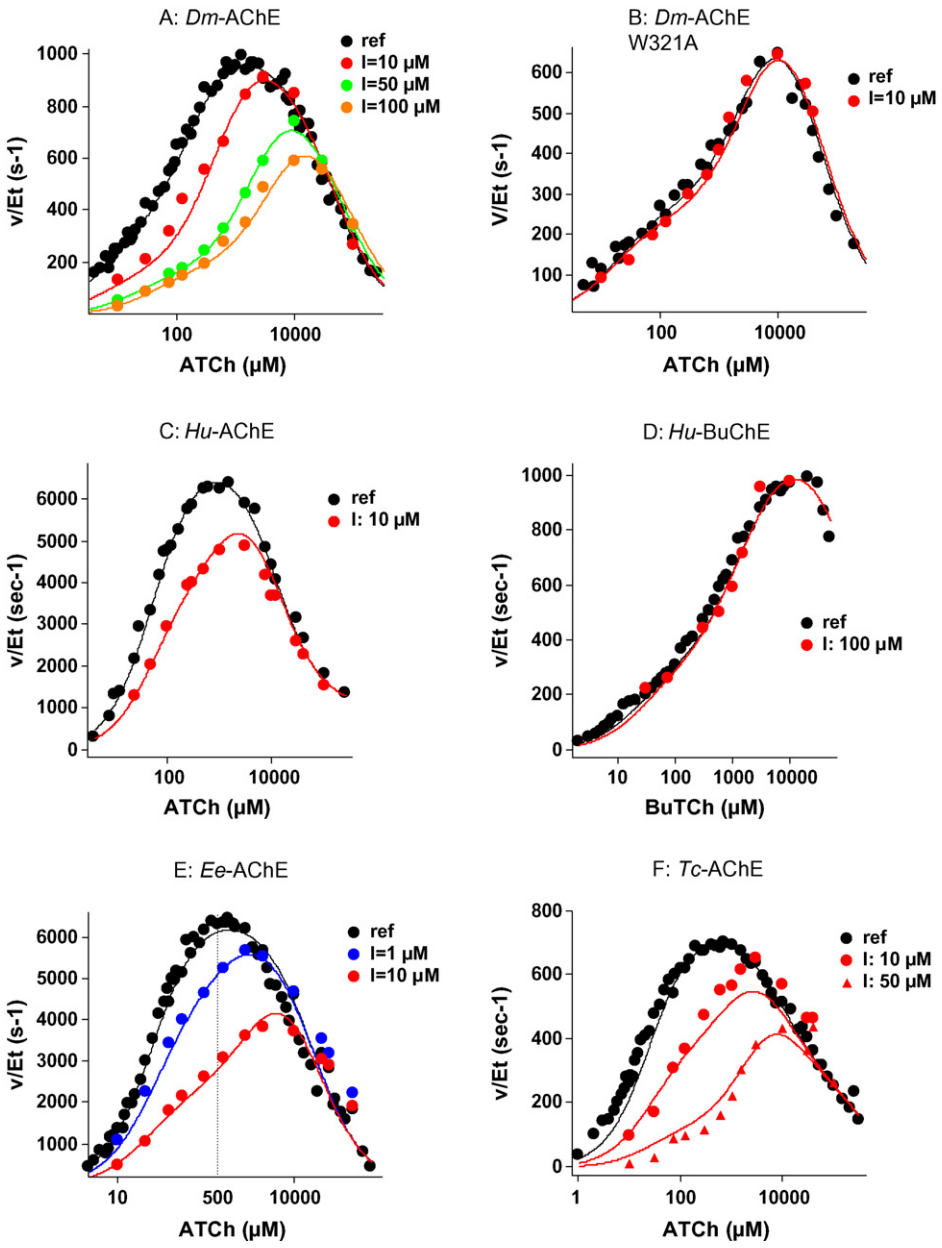


Fig. 1. Representation of the AFB1 inhibition curves for six AChE species. (A) *Drosophila melanogaster*-AChE. (B) *Drosophila melanogaster*-AChE peripheral site mutant. (C) *Human*-AChE. (D) *Human*-BuChE. (E) *Electric eel* AChE. (F) *Torpedo californica* AChE. ATCh concentration in µmol per litre; v/Et: substrate turnover, velocity/total enzyme concentration in s^{-1}.

measurements cannot be made because of the substrate depletion by enzyme hydrolysis during the experiment. Thus, a substrate concentration of 500 µM was chosen to obtain the maximum of inhibition (Fig. 2). Since detection values for inhibition by toxins can be quite variable, at least 20% inhibition is required to be sure of the authenticity of the results obtained which corresponds to a concentration of 3 µM AFB1.

4. Discussion

4.1. Binding site of aflatoxin B1

As previously discussed, AFB1 inhibits AChEs by binding at the peripheral site, located at the entrance of the active site gorge (at the tryptophan residue). It obstructs the entrance of the active site, substrate cannot enter to the catalytic site and choline cannot exit as proposed by the steric blockade model (Szegletes et al., 1998).

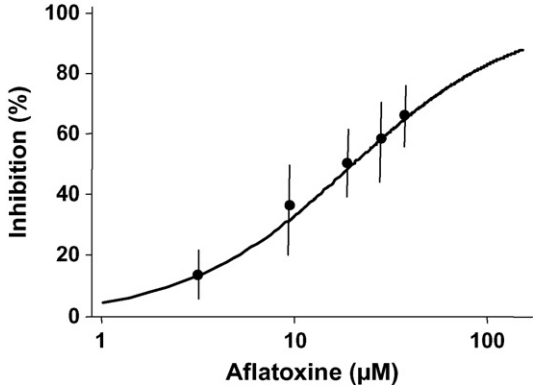


Fig. 2. Biosensor sensitivity. Inhibition of Ee-AChE immobilized on electrode as function of alfatoxin concentration and amount of enzyme immobilized on the electrode (1 mU corresponds to 1 fmol enzyme).

Table 1
Kinetic parameters estimated according to Scheme 1 for four AChE species (standard error).

	Substrate (ATCh)							Aflatoxin B1		
	K_p (μM)	K_i	K_{iL}	k_2 (s^{-1})	k_3 (s^{-1})	a	b	K_{ip} (μM)	c	d
Dm-AChE	159 (24)	4.06 (2.43)	190 (37)	44,563 (25,714)	347 (46)	3.82 (0.41)	0.078 (0.041)	2.39 (0.44)	1.1 (0.202)	0.023 (0.009)
Hu-AChE	543 (101)	0.018 (0.005)	9.23 (6.6)	14,375 (2,182)	13,471 (2,171)	1.6 (0.36)	0.01 (0.003)	5.1 (7.8)	3.28 (0.76)	0.155 (0.041)
Ee-AChE	319 (37)	1.29 (0.06)	66.4 (3.7)	73,957 (3,676)	7,165 (200)	1.52 (0.129)	0,077 (0.014)	0.35 (0.05)	0.34 (0.03)	0.53 (0.07)
Tc-AChE	449 (13)	1.7 (0.25)	269 (166)	24,000 (160)	836 (19)	0.93 (0.16)	0.017 (0.014)	9.8 (0.4)	0 (0.7)	0.017 (0.014)

These results emerged from the following observations: (1) Addition of a binding step of AFB1 inside the active site did not improve the description of inhibition, i.e. did not improve the fitting, which suggests that AFB1 does not slide to the catalytic site. (2) Mutation of Trp321 to alanine in Dm-AChE abolishes inhibition at 10 μM and 100 μM AFB1 does not inhibit Hu-BuChE. We may hypothesize that aflatoxin B1 does not enter into the active site because it is too big, especially when considering that its hydrophilic shell may further enlarge its size. Thus, aflatoxin is added to the list of ligands which bind on the peripheral site of cholinesterases such as propidium, gallanine or fasciculin (Bourne et al., 2003).

4.2. Comparison of binding constants with toxic doses to humans

One of the goals of this study was to address if cholinesterase inhibition plays a role in aflatoxicosis. Since no precise data of toxic amounts are available for humans, the of aflatoxin B1 for peripheral site (K_{ip}) of Hu-AChE (13 μmol/l) was compared to the known LD_{50} values for different animal species ranging between 1 and 32 μmol/kg bodyweight (Wogan, 1966). Actually, this value is in the same range, suggesting that acetylcholinesterase may be inhibited *in vivo* during aflatoxicosis. However, AFB1 is not homogeneously distributed in the body, but it accumulates in the liver, although neither reaches the muscles, nor the brain that contain the highest AChE density (Wogan, 1966). Aflatoxin B1 has been reported to inhibit other enzymes such as trypsin, amylase, lipase or cytochrome P450 (Guerre et al., 1997; Uwaifo, 1980) and aflatoxicosis has been associated with reduced activity levels of several enzymes (Osborne and Hamilton, 1981). Furthermore, some aflatoxin B1 metabolites are known to form adducts with proteins by conjugation with the ε-amino group of lysines. Thus, acetylcholinesterase represents one of few investigated enzymes whose activity is affected by AFB1 binding. Consequently, inhibition of acetylcholinesterase may not be significantly involved in aflatoxicosis etiopathology since hepatic, nutritional and immunologic symptoms are predominant.

4.3. Sensitivity of the biosensor

Attempting to create an AFB1 biosensor in the environment, the best conditions for a strong inhibition of Ee-AChE with small amounts of AFB1 were investigated.

Using the AChE biosensor, the minimum concentration of AFB1 detectable was 3 μM, corresponding to 1 mg/L. Regarding the fact that the European limit lays at 2 ppb (2 μg/kg) in grain and peanuts, it would be necessary to extract AFB1 from 0.5 kg of the crop sample and to concentrate the extract down to 1 mL or less. Thus, the sensitivity of Ee-AChE should be increased by mutagenesis to create an optimized biosensor able to facilitate the detection of AFB1 in the environment, compared to ELISA-immunoassays or chromatographic methods.

5. Conclusions

This paper confirms previous report claiming the inhibition of AChE by aflatoxin B1 (Cometa et al., 2005; Arduini et al., 2007). However, this inhibition does not seem to be involved in aflatoxicosis. The inhibition mechanisms of AFB1 were investigated using steady state kinetic measurements and *in vitro* mutagenesis. Experimental data suggests that inhibition comes from the binding of AFB1 on the peripheral site, located at the entrance of the active site. Binding constant has been found to depend on the enzyme source. Among studied enzymes, electric eel enzyme was the most sensitive with an affinity estimated to 0.35 μM. This enzyme was used for the development of a field-compatible biosensor. Its detection limit was estimated to 3 μM.

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