A RAPID METHOD FOR AFLATOXIN B_1 DETECTION BASED ON ACETYLCHOLINESTERASE INHIBITION

5.1 Introduction

Cereals and a wide variety of food products are susceptible to damage from mould during pre- as well as post-harvest stages of production. The family of aflatoxins, which can be produced by several species of the mould *Aspergillus* (*Aspergillus flavus, Aspergillus parasitucus* and the rare *Aspergillus nomius*), has by now been shown to represent a significant class of mycotoxins because of their documented impact on both human and animal health and on economic aspects of international trade involving food and animal feeds [1,2]. Due to its capacity for covalent binding to DNA and proteins [3,4], aflatoxin B_1 (AFB_1) is the most acutely and chronically toxic member of the aflatoxin family. It is also the most widely found of the aflatoxins (AFB_2, AFG_1, AFG_2, and the metabolic product $AFM_{1)}$. The legal limits set for AFB_1 or for total aflatoxins vary significantly from country to country (e.g. for total aflatoxins from 0 to 50 ng/g) [5].

In light of this overall scenario, several important issues require a response. Most studies of aflatoxins have focused on their chronic toxic effects as carcinogens, mutagens, and hepatotoxins. So regulatory limits have been established as a means to protect human and animal health and the food supply. At the same time, there are limited analytical methods available for the determination of aflatoxins within this strict regulatory context. The current reference methods are primarily chromatographic, relying on methods such as high performance liquid chromatography (HPLC) [6-10]. A promising alternative approach involves the use of enzyme linked-immunoassay (ELISA) [11,12]. While they have often demonstrated the required sensitivity and acceptable specificity, these assays need specific antibodies and, indirectly, the use of animals in order to produce these “receptors”.

Given the demonstrated toxicity of aflatoxins, their potential widespread presence in the food chain, and the economic costs of contaminated lots, much attention has also been focused on a complementary approach, the development of practical screening assays that could be used for monitoring at all stages of food and feed production, so that contaminated ingredients can be identified or rejected before feeding to animals or humans. Such methods must be rapid, sufficiently sensitive, and applicable in the field in order to allow the necessary corrective actions to be taken in a timely fashion. To respond to these various issues, enzymatic methods have shown promise in some cases as an alternative to classical methods to achieve faster and simpler detection of some environmental pollutants such as pesticides, heavy metals, etc. [13-19]. In the case of the aflatoxins, this approach pointed to either an enzyme that metabolizes AFB_1 into a detectable compound or an enzyme that can be inhibited by AFB_1. It can be noted that, as for biosensors, the use of biological molecules (enzymes or receptors) in analytical schemes can be particularly advantageous if the action of the analyte is specifically related to the mechanism of toxicity towards humans and animals. In this perspective, recent work has provided insight into the acute effects of the aflatoxins on the gastrointestinal, respiratory, cardiovascular, and central nervous systems of both humans and animals [20-24]. In particular, AFB_1 could be shown to evoke contractile responses in the rumen intestine [25] and on isolated guinea pig ileum [26]. These results demonstrated that the aflatoxin induces its contractile effect indirectly through the cholinergic system by stimulating acetylcholine release. Moreover, the work of Egbunike and Ikegwuonu [27] suggested that AFB_1 changes acetylcholine turnover and hence cholinergic transmission in rat brain and adenohypophysis. In a recent study, Cometa et al. [28] have analyzed the inhibition of acetylcholinesterase (AChE) by AFB_1, a key enzyme in nervous impulse. The AFB_1 inhibition on AChE extracted from mouse brain was studied and the implications in terms of kinetic mechanism and toxicity discussed. In this study, it was first shown that AFB_1 inhibits the AChE from *electric eel*. On the basis of this effect, a method to

quantify AFB_1 by measuring the decrease in enzyme activity, determined using Ellman's spectrophotometric method, after exposure of the enzyme to AFB_1 was proposed and optimised.

5.2 Experimental section

5.2.1 Apparatus and reagents

All chemicals from commercial sources were of analytical grade. Acetylcholinesterase (AChE) from *electric eel* (EC 3.1.1.7), butyrylcholinesterase (BChE) from *equine serum* (EC 3.1.1.8), S-butyrylthiocholine chloride,

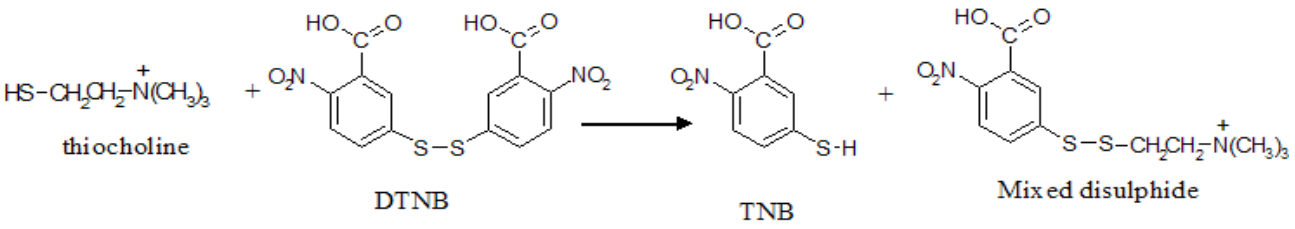
acetylthiocholine chloride (ACTh), DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) were obtained from Sigma Chemical Company (St. Louis, MO). The AChE from *Drosophila melanogaster* wild type and mutants were obtained from Marty's group (University of Perpignan, France). The AChE from *Drosophila melanogaster* were dialyzed before use. Aflatoxin B_1, B_2, G_1, G_2, M were purchased from Vinci–Biochem, Italy.

Stock solutions of AFB_1 were preparated in methanol and their concentration was confirmed with HPLC method using the official AOAC method with some modifications [11]. Different concentrations of aflatoxin were obtained by diluting the stock solution with methanol.

For spectrophotometric measurements an Unicam 8625 UV/VIS spectrophotometer was used.

5.2.2 Measurement of cholinesterase activity

The AChE activity was evaluated measuring the product of the enzymatic reaction. The ACTh was chosen as substrate and the thiocholine, enzymatically produced using AChE, was evaluated using spectrophotometric Ellman's methods. The thiocholine, is measured following the scheme 1: DTNB (5,5'-dithiobis-2-nitrobenzoic acid) reacts with thiocholine to give TNB (2-nitro-5-thiobenzoic acid), a yellow product with a maximum absorbance peak at 412 nm [29].



Scheme 5.1. Reaction between DTNB (5,5'-dithiobis-2-nitrobenzoic acid) and thiocholine to give TNB (2-nitro-5-thiobenzoic acid)

For thiocholine measurement, 900 μL of phosphate buffer solution (0.1 M, pH=8), 100 μL of 0.1 M DTNB, 40 mU mL^{-1} of enzyme (AChE) and ATCh 0,4 mM were put in a spectrophotometric cuvette. Absorbance was measured after 3 minutes and the AChE activity was evaluated, using the Lambert–Beer law and known the molar extinction coefficient of TNB (λ= 13,600 M^{-1} cm^{-1}) [29].

5.2.3 Measurement of the effect of methanol on Ellman's reaction

The methanol is often used to extract the AFB_1 from many contaminated agricultural samples [30-32], then the methanol was chosen as organic solvent to solubilize AFB_1 in our experiments.
In the experimental procedure the effect of methanol was evaluated on the Ellman's method. In this perspective, the cisteamine was taken as a representative thiol for this investigation since it is commercially available whereas thiocholine has to be enzymatically produced [33]. An aliquot of 900 μL of phosphate buffer solution (0.1 M, pH 8.0) containing different percentages of methanol (from 0% to 90% v/v) and cisteamine to a final concentration of 1.9 10^{-5} M were added in a cuvette. The absorbance was measured using the procedure described above.

5.2.4 Measurement of the effect of methanol on AChE activity

The effect of the methanol on the enzyme activity was also evaluated. An aliquot of 900 μL of phosphate buffer solution (0.1 M, pH 8.0) containing different percentage of methanol (from 0% to 90% v/v) and a known amount of AChE was added. After the substrate was added, the residual enzymatic activity was evaluated by using the procedure described above. Inactivation of enzyme by exposure to organic solvent was calculated by using Equation (5.1):

$$I\% = (A_0 - A_i)/A_0 \qquad (5.1)$$

where I% is % of enzyme inactivation due to methanol, A_0 is absorbance obtained in aqueous solution, and A_i is absorbance obtained in aqueous solution with methanol.

5.2.5 Measurement of the effect of methanol on AChE inhibition

The effect of the methanol on the AFB_1 inhibition on AChE was also evaluated. An aliquot of 900 μL of phosphate buffer solution (0.1 M, pH 8.0) containing known amount of AChE and various percentage of methanol (from 0% to 50% v/v) in the absorbance or presence of different concentrations of AFB_1 was added. After the substrate was added and the residual enzymatic activity was evaluated by using the procedure described above. AFB_1 inhibition on enzyme was evaluated by using Equation (5.1), where I% is % of AFB_1 inhibition on enzyme, A_0 is absorbance obtained in aqueous solution with methanol, and A_i is absorbance obtained in aqueous solution with methanol and AFB_1. The measurement of A_0 adding methanol is necessary to considered also the AChE inactivation by methanol.

5.2.6 Measurement of stock solution aflatoxin

For aflatoxin measurements, the same procedure described above was used. The inhibition due the toxin was calculated by using Equation (5.1) where I% is % of inhibition, A_0 is absorbance obtained by using methanol, and A_i is absorbance obtained by using this analyte in methanol, being the its favorite solvent.

5.2.7 Spiked barley sample preparation and extraction

Non-infected barley seeds (from local market) were first ground in a household blender at high speed for 1 min. 5 g of ground barley samples were spiked with 200 µL of AFB_1 solution at several concentrations (in order to have in barley 100, 120, 150 ng g^{-1}). The samples were fully mixed using auto-vortex for 1 min and extracted with 25 mL of extraction solvent (70 % methanol: 29 % phospate buffer: 1% dimethylformamide by shaking for 15 min, then the extract was separated from the insoluble materials by centrifugation for 5 min at 4000 rpm. After the separation of the two layers, the aqueous layer was separate and concentrated in a rotary evaporator to remove the solvent. The dryed matrix mixture was redissolved in 1 mL of methanol and the obtained solution was filtered on glass fiber membrane (1 µm). The filtered solution was used for AFB_1 spectrophotometric assay.

5.2.8 Measurement of AFB_1 in barley

For AFB_1 measurements, the same procedure described above was used. The inhibition due the AFB_1 in barley was calculated by using Equation (5.1) where I% is % of inhibition, A_0 is absorbance obtained by adding extract of barley without AFB_1, and A_i is absorbance obtained by adding extract of barley with AFB_1.

5.3 Result and discussion

5.3.1 Aflatoxin inhibition on different ChEs

Since our objective is to device an assay that can measure AFB_1 at lowest concentrations, a survey of cholinesterases (ChEs) from various sources was carried out to have the highest degree of inhibition by AFB_1. For this purpose, we have studied the AFB_1 inhibition using AChE from *electric eel*, butyrylcholinesterase (BChE) from *equine serum*, and AChE from *drosophila melanogaster* wild type and mutants. In particular, two different AChE engineered from *Drosophila melanogaster* (B24 and B394) were used. The AChE from *electric eel*, was the most sensitive: in fact IC_{50} (IC_{50} equal to amounts of inhibitor which gave 50% enzyme inhibition) was obtained with 60 ng mL^{-1} of AFB_1. The inhibition by AFB_1 on BChE from *equine serum* was also investigated. In the case of BChE, at a concentration of 60 ng mL^{-1} of AFB_1 no inhibition was observed while only 10% inhibition was obtained using 1,4 mg mL^{-1} of AFB_1. This demonstrated the very low affinity between AFB_1 and BChE. The AFB_1 inhibition on AChE from *Drosophila melanogaster* wild-type and two different mutants (B24 and B394) was tested. The AChE was engineered from laboratory by Marty's and Fournier's group to increase the sensitivity towards the pesticides [34-36]. The degree of inhibition obtained with AFB_1 60 ng mL^{-1} using AChE wild-type, B24 and B394 were 20%, 17%, 21% respectively. Overall, the results obtained demonstrated that the AChE from *electric eel* had the highest sensitivity to AFB_1 among the cholinesterases previously tested and was also considerably more sensitive than the unpurified AChE from *mouse brain* tested by Cometa et al. which showed IC_{50} at 10 mg mL^{-1} [28].

In order to understand better the type of AFB_1 inhibition on AChE mainly to select the best analytical procedure, kinetic studies were carried out.

5.3.2 Mechanism of the AChE inhibition by AFB_1

5.3.2.1 Measurement of AChE activity

The first step was the study of the K_M of the enzyme (AChE from *electric eel*) using ACTh as substrate. The AChE activity was determined using Ellman's test. 40 mU mL^{-1} of AChE and 3 min of reaction were sufficient to perform the spectrophotometric measurements. Different concentrations of substrate were evaluated in dynamic range 0.1-5 mM of ACTh. In this range of ACTh the values of absorbance from 0.050±0.002 to 0.80±0.02 were observed.

Enzymatic analysis of ATCh hydrolysis using AChE yielded the following kinetic values: K_M 0.33 mM and V_{max} 0.26 mmol $l^{-1}min^{-1}$ using a Lineweaver Burk's plot (data not shown).

5.3.2.2 The reversibility of the AChE inhibition by AFB_1

It was essential to establish certain aspects of the inhibitory mechanism in order to determine the suitability of a given enzyme for a screening assay. Since little is known about the inhibition by AFB_1 [20,28], the mechanism of AChE inhibition by the mycotoxin AFB_1 was first investigated.

In the first instance, whether the inhibition is of a reversible or irreversible type is crucial, since for irreversible inhibition, it becomes important to use a low enzyme concentration in order to obtain a low limit of detection, as in the case of pesticide detection [33,37]. On the other hand, for reversible inhibition, no change in degree of inhibition should be observed using different enzyme concentrations [38]. The degree of inhibition at fixed concentration of AFB_1 (60 ng mL^{-1}) using various concentrations of AChE was determined. The enzyme concentrations used for this experiment were: 70, 40 and 7 mU mL^{-1} and the degrees of inhibition obtained were 45±3%, 50±4% and 47±3% respectively. These results (essentially no change in degree of inhibition) seem to support the hypothesis that the inhibition of AChE by AFB_1 follows a reversible mechanism.

To confirm this hypothesis, a study of the incubation time with the inhibitor was performed. While for the case of irreversible inhibition, an increase in incubation time is required to reach a

low detection limit, for reversible inhibition, a longer incubation time does not lead to an increase in the degree of inhibition. Then, the effect of incubation time (the time of reaction between the inhibitor, $AFB_{1,}$ and AChE) was also evaluated between between 0 to 30 min obtaining a degree of inhibition equal to 48±3%.

Given that the incubation time has no effect on degree of inhibition, that is, that the interactions were rapid and reversible, there was no need for a prior incubation of the enzyme with inhibitor before substrate was added to start the reaction.

5.3.2.3 Study of the type of the AChE inhibition by AFB_1

After demonstrating the reversibility of this inhibition, experiments were performed to evaluate whether the inhibition was competitive, uncompetitive, non-competitive or mixed.

AChE activity was determined using ATCh concentration over the range from values near $K_M/4$ to 4 K_M either was in the absorbance or presence of fixed AFB_1 concentrations (60 ng mL^{-1}). The degree of inibition (47±5) % does not change with substrate concentrations. This study indicates that the inhibition is not competitive in nature. In fact, pure competitive inhibition is characterized by the increase of inhibition degree as the substrate decreases.

To understand if the inhibition is uncompetitive, non-competitive or mixed type, the AChE activity was determined with the previous range of ATCh concentrations (from values near $K_M/4$ to 4 K_M) either were in the absorbance or presence of different AFB_1 concentrations.

The analysis of the linear transformation of Michaelis-Menten equation, Lineweaver-Burk plot (fig. 5.1) confirmed that the lines are not parallel and thus the inhibition is not uncompetitive. In this way, the mechanism of inhibition could be non-competitive or mixed type.

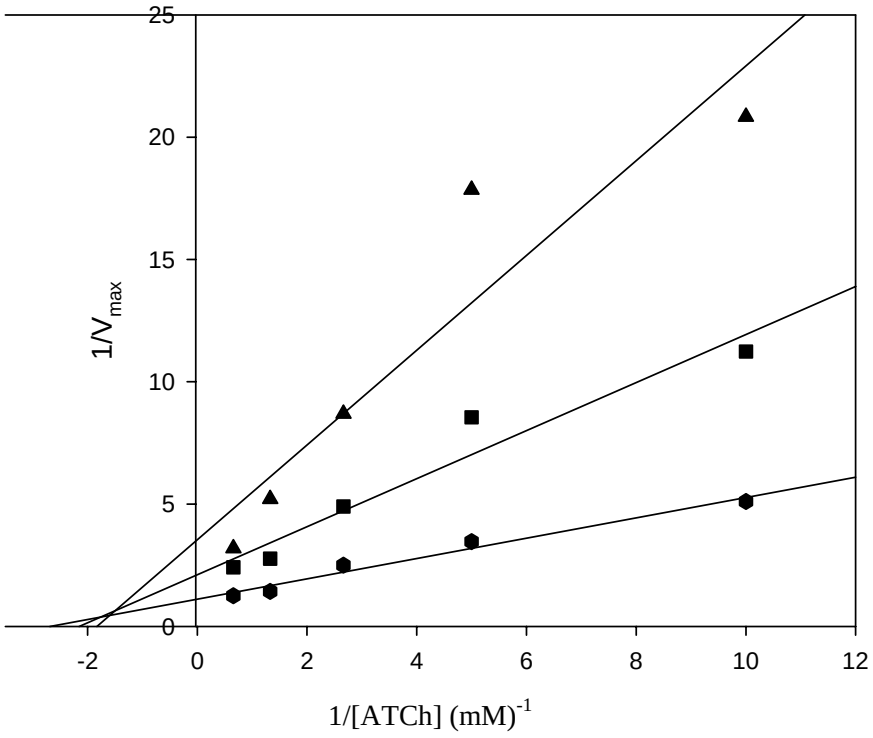


Fig. 5.1 Lineweaver-Burk plot representing reciprocals of the initial enzyme velocity vs. ATCh concentration without (⬢) and with various concentrations of AFB_1 (75 ■ and 300 ▲ ng mL^{-1} of AFB_1). AChE=40 mU mL^{-1}, 3 min of reaction, phosphate buffer 0.1 M, pH 8.

In the case here of the AChE from *electric eel*, the lines pass through different points on the ordinate and intersect at a point slightly displaced from the abscissa in the second quadrant, thus we can suppose that the inhibition seems a mixed type.

In the case of the mixed type inhibition, the inhibition can be regarded conceptually as being composed of a competitive and non-competitive inhibition. The dissociation constants for inhibitor differ from each other, depending on whether they refer to the free enzyme or the complex [38]. The costants K_i (inhibitor dissociation constant for binding to the free enzyme) and αK_i (inhibitor dissociation constant for binding to the enzyme-substrate complex) were calculated

using kinetic equations for mixed inhibition. For K_i calculation the equation $K_{Mapp}/V_{max\ app}= K_M(1+[I]/K_i)$ was used with 75 ng mL^{-1} of AFB_1 obtaining a value of K_i equal to 61 ng mL^{-1}. The αK_i was calculated equal 130 ng mL^{-1}, from the equation $1/V_{max\ app}= (1/V_{max} +([I]/ \alpha K_i\ V_{max})$ using 75 ng mL^{-1} of AFB_1. Our hypothesys of the mixed inhibition type, seems similar to the hypothesis supposed by Cometa et al. [28] for the AFB_1 inhibition on AChE unpurified from *mouse brain,* but further investigations in order to deeply understand the mechanism of the inhibition are under study.

5.3.3 Optimization of the AFB_1 assay based on AChE inhibition

In order to develop an effective assay for AFB_1 determination based on the decrease in enzyme activity, the spectrophotometric Ellman's method for the determination of AChE activity had to be adapted and optimised, taking into consideration parameters such as substrate concentration, solvent, pH, etc.

As noted above, characteristics of the inhibition made possible to adjust the enzyme and substrate concentration of the assay mixture to achieve suitable assay times and levels of inhibition. An enzyme concentration of 40 $mUmL^{-1}$ was chosen because it permitted the achievement of reasonable optical densities from the Ellman's reaction in only 3 min. Furthermore, at a constant concentration of AFB_1 60 ng mL^{-1}, with different concentrations of substrate (0.1-1.6 mM), the degree of inhibition (47±5%) was found to be constant. A substrate concentration of 0.4 mM was chosen for the rest of the work.

Given that the incubation time has no effect on degree of inhibition, because for the reversible inhibition, there was no need for a prior incubation of the enzyme with inhibitor before substrate was added to initiate the reaction. For this reason, no incubation time was required for the rest of work.

5.3.4 Effect of pH

As reported in our previous work [39], the best buffer and pH value for AChE is phosphate buffer 0,1 M at pH=8.0 To evaluate AChE inhibition by AFB_1 as a function of pH, the AChE, ATCh and AFB_1 60 ng mL^{-1} was added in buffer at different pHs and after 3 min the absorbance was measured. The degrees of inhibition obtained are shown in fig. 5.2. We observed the highest degree of inhibition at pH 8.0 with phosphate buffer, and a rapid decrease at pH 9.0 was observed in Tris buffer. Thus, a phosphate buffer at pH 8.0 was chosen for further experiments.

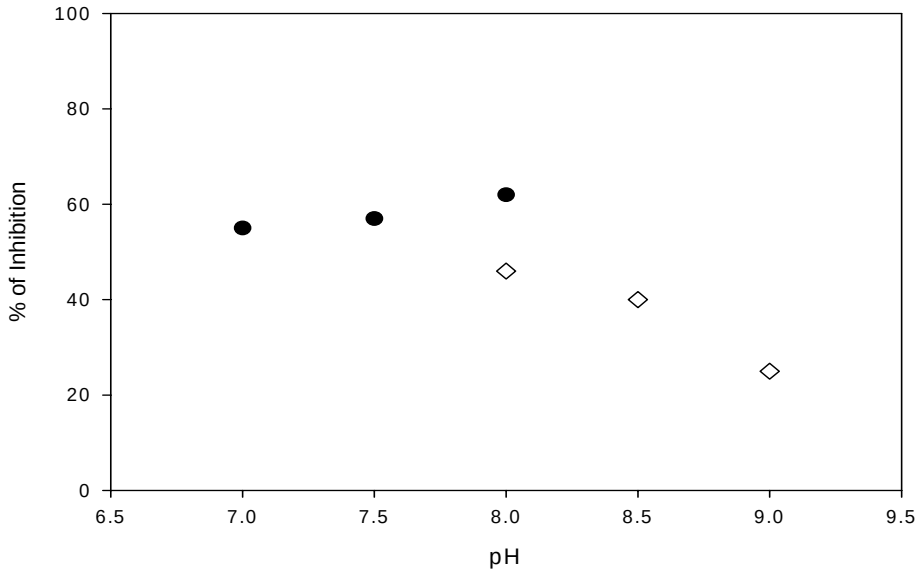


Fig. 5.2 Effect of pH on AChE inhibition by 60 ng mL^{-1} of AFB_1. AChE=40 mU mL^{-1}, 3 min of reaction, ATCh= 0,4 mM. At pH 7.0, 7.5 and 8.0 the phophate buffer 0,1 M was used while at pH 8.0, 8.5 and 9.0 a Tris buffer 0,1 M was chosen.

5.3.5 Effect of temperature on enzyme activity in storage condition

Storage stability, or shelf life, refers to an enzyme's maintaining its catalytic properties in the period between manufacture and eventual use. A common inactivation model for an enzyme involves the unfolding of the polypeptide's native tertiary structure. The unfolded protein may refold to the native conformation or undergo some further change leading to permament inactivation. Temperature is a parameter that influences the enzyme stability [40]. As it can be seen in fig. 5.3, AChE maintained at room temperature (25°C) was gradually inactivated, while no decrease of enzymatic activity was observed when the enzyme was maintained at 4°C. Thus AChE was stored at 4°C.

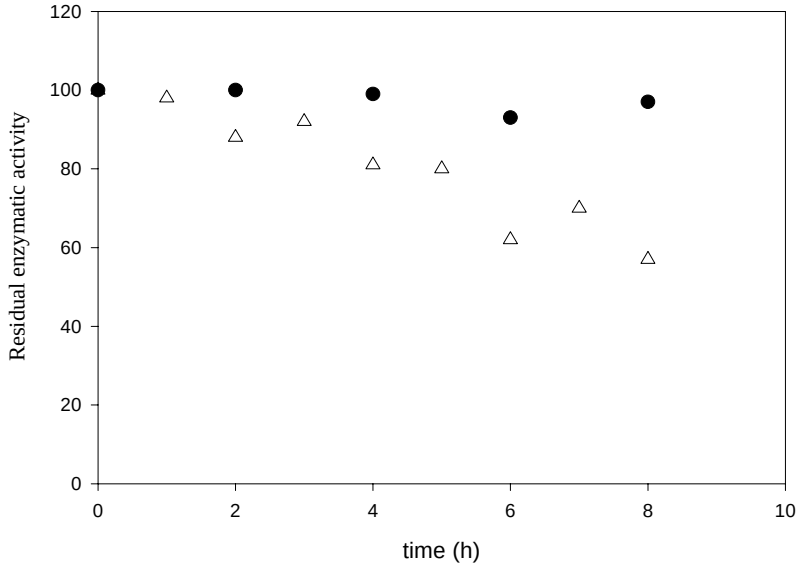


Fig. 5.3 Effect of temperature on AChE storage conditions. AChE=40 mU mL^{-1}, 3 min of reaction time, ATCh= 0,4 mM. The residual activity was evaluated for AChE maintained at room temperature RT (△) and at 4°C (●).

5.3.6 Study of methanol effect

In the previous experiments, the AFB_1 inhibition on AChE was carried out in methanol because AFB_1 is insoluble in completely non-polar solvents and soluble in slightly polar solvents. The AFB_1, in fact, is normally extracted using mixtures of organic solvents such as methanol, acetonitrile, chloroform or acetone. The methanol has previously been used to extract AFB_1 from many contaminated agricultural samples [30-32] and was chosen as the organic solvent to solubilized AFB_1 in the our experiments. In a perspective to adopt the assay for AFB_1 detection in barley the effect of methanol was evaluated in the assay.

Since Ellman's method was to be utilized to monitor the thiocholine enzymatically produced, a first step was to evaluate the direct methanol effect on thiols determination. Cisteamine was taken as a representative thiol for this investigation since it is commercially available whereas thiocholine had to be enzymatically produced. The reaction between cisteamine ($1.9\ 10^{-5}$ M) and DTNB (Ellman's reagent) was evaluated in the presence of different percentages of methanol, in the range from 0% to 90%, mixed with buffer. Since the same value of absorbance (0.230 ± 0.007) was obtained when the methanol percentage in phosphate buffer was 0%, 50%, 60%, 70% and 80%, it was concluded that methanol had no effect on thiols detection using Ellman's method.

In terms of the enzyme's sensitivity to solvent effects, it is known that the AChE is inactivated by organic solvents such as DMSO (dimethylsulfoxide), toluene, dichloromethane, chloroform [39,41,42]. For this reason, AChE inactivation by methanol was investigated. Fig.5.4a shows that the AChE activity decreased in parallel with an increase in the amount of methanol. At the 50% methanol (in phosphate buffer), the AChE activity decreased by 30%. If the percentage of methanol was increased to 80%, the inactivation seemed to level off at 60% of the original activity.

Using lower concentrations of methanol, it was found that in the range 0-5% of methanol in buffer no inactivation was observed. Thus, in setting up the inhibition assay system, a methanol percentage lower than 5% was always used.

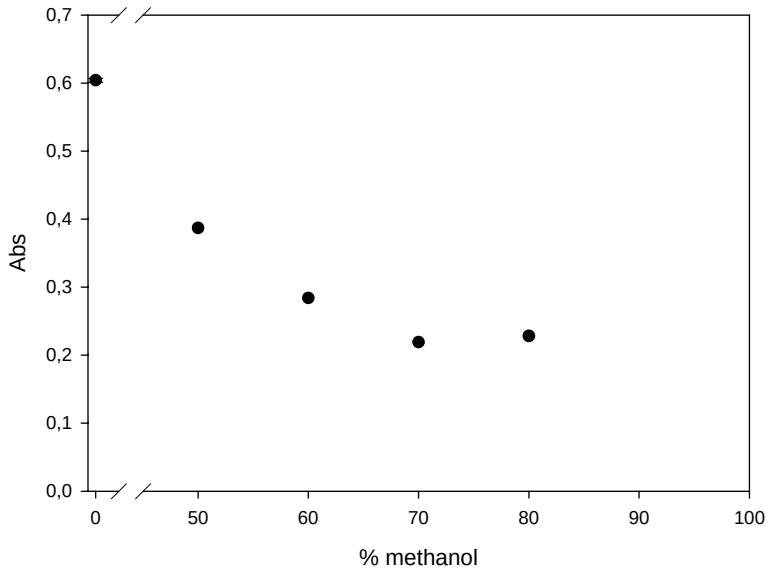


Fig. 5.4a Effect of methanol on AChE activity. AChE= 40 mU mL^{-1}, 3 min of reaction, acetylthiocholine= 0.4 mM, phosphate buffer 0.1 M, pH 8.0.

The effect of the methanol on the inhibition of AChE by AFB_1 was also investigated using the optimised assay conditions (fig.5.4b). This study was carried out by performing the assay in the presence of different amounts of methanol (from 0% to 50%). The degree of inhibition was calculated using the equation 5.1, where I% is % of inhibition, A_0 is the absorbance obtained when the cuvette contains phosphate buffer, enzyme, substrate and Ellman's reagent in the presence of a given percent of methanol and A_i is absorbance obtained by adding 60 ng mL^{-1} of

AFB_1 to the same system used for A_0 measurement. In this way the inactivating effect of methanol on the enzyme is controlled.

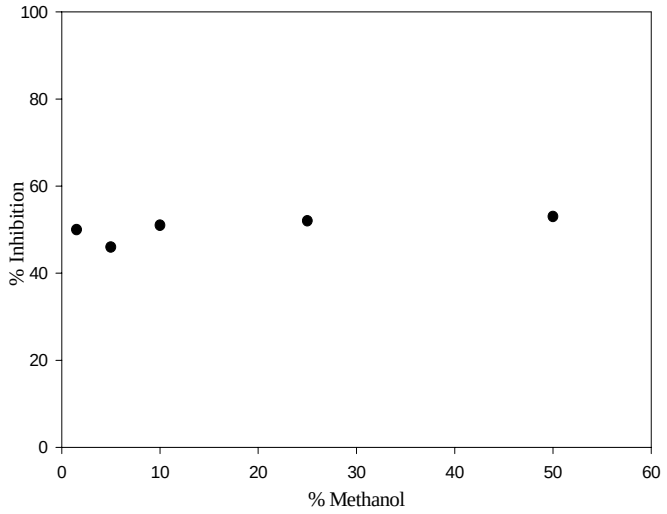


Fig. 5.4b Effect of methanol on AChE inhibition by AFB_1. AChE= 40 mU mL^{-1}, 3 min of reaction time, ATCh concentration=0.4 mM, AFB_1 concentration=60 ng mL^{-1}, phosphate buffer 0.1 M, pH 8.0.

The degree of inhibition by 60 ng mL^{-1} of AFB_1 equal to (50±3%) in the presence of different amounts of methanol (from 0% to 50%) was obtained. These results demonstrate that even the highest percentage of methanol has no effect on the degree of AChE inhibition by AFB_1 though this amount of methanol in buffer decreased the control level of AChE activity. This result means that it is possible to determine AFB_1 using a percentage of methanol as high as 50%, that is diluting the AFB_1 standard solution (preparated in pure methanol) two times.

5.3.7 Calibration curve

From an analysis of inhibition by AFB_1 over the concentration range 10-1000 ng mL^{-1} (data not shown), it was found that the response for the optimised assay was linear in the range between 10-60 ng mL^{-1}. Five successive calibration curves were performed and the results showed a linear correlation: $y=(13,4\pm0,9)+(0,602\pm0,009)x$, where y is degree of inhibition and x is AFB_1 concentration with r^2 equal to 0,955. The lower limit of the linear range, definited as the concentration giving an inhibition of 20%, was 10 ng mL^{-1} of AFB_1 while the IC_{50} was 60 ng mL^{-1} of AFB_1 .

5.3.8 Cross-reactivity

To evaluate the selectivity of our proposed assay, IC_{50} values were determined using the other aflatoxins (AFB_2, AFG_1, AFG_2 and AFM_1) for comparison with that for AFB_1. As shown in table 5.1, both AFB_1 and AFB_2 provoked considerable inhibition of AChE while both AFG_1 and AFG_2 showed limited capacity to inhibit the enzyme. The results for AFM_1 were intermediate with an IC_{50} of 175 ng mL^{-1}.

The structural difference between AFG and AFB forms is the presence of a cyclohexane in structure of AFG, while in AFB there is a cyclopentane. This difference probably may affect the interaction between aflatoxin and its binding site on the catalytic unit of AChE.

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Aflatoxin	IC_{50} (ng mL^{-1})
AFB_1	60
AFB_2	72
AFG_1	850
AFG_2	310
AFM_1	175

Table 5.1 Concentration of various aflatoxins giving 50% enzyme inhibition (IC_{50}). AChE=40 mU mL^{-1},3 min of reaction time, ATCh=0.4 mM

5.3.9 AFB_1 measurement in fortified barley samples

The AChE inhibition assay was then applied for the determination of AFB_1 using spiked barley samples in order to test its performance in a real matrix. Firstly, the absorbance of the sample in absorbance of Ellman's reagent was evaluated. An absorbance equal to 1,760 was observed by a sample dilution in buffer of two times. Different dilutions of the sample in buffer were evaluated in order to obtain a lowest matrix effect. It was necessary to make dilution (1:15 v/v) of the sample to decrease the absorbance of the sample to value 0.100. This dilution factor (15) was adopted for the recovery study.

The extraction efficiency of the toxin from barley was calculated using samples spiked before the extraction with known amounts of the toxin. A 200 μL of AFB_1 standard solution at different concentrations was added to 5 g of blank barley in order to obtain 100, 120 and 150 ng g^{-1} of barley sample. For each AFB_1 level, three samples were independently processed and each

sample was analysed in triplicate. On the basis of the calibration curves prepared in barley extract it was possible to calculate the extraction efficiency (Table 5.2) of the analyte.

The results show that the procedure adopted for extraction of AFB_1 from barley is both quick and shows recovery of 99± 5%.

AFB_1 added (ng mL^{-1})	**AFB_1 found (ng mL^{-1})**	**% Recovery**
150	152	101 ± 2
120	117	98 ± 3
100	99	99 ± 9

Table 5.2 Recovery studies of spiked barley samples using assay for AFB_1 based on AChE inhibition (concentration in barley).

5.4 Conclusion

A new method for AFB_1 detection was developed. The determination of AFB_1 is based on its capacity to inhibit acetylcholinesterase (AChE) from *electric eel* and it makes use of the Ellman's spectrophotometric method to measure the enzyme activity in the absence and presence of analyte. In fact, within a few minutes it is possible to obtain results suitable for a rapid screening of the prepared samples.

A preliminary investigation of the kinetics and inhibitory mechanism indicated a mixed type. It is interesting to note that the binding of AFB_1 to the *electric eel* enzyme is about three orders of magnitude stronger than that found for the mouse brain AChE [28] and this behavior allowed AFB_1 to be detected, through an enzyme inhibition based assay, at levels near the regulatory limit

of a few ppb. Finally, while the results presented here showed that the assay detected AFB_1 and AFB_2 with similar sensitivity, this does not necessarily compromise its value as a screening assay. If it can be demonstrated that the inhibition of a given AChE by aflatoxins bears a direct relation to their toxic effects (eg. cholinergic ones) then it becomes an advantage relative to the ultimate objective of effectively detecting the presence of harmful substances in order to remove contaminated lots from the food production chains.

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