Acetylcholinesterase from Curimatã Fish Brain (Prochilodus Brevis) as Potential Biocatalyst for Voltammetric Biosensor Construction

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Received: August 23, 2018; Published: August 28, 2018

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Abstract

Background: The AChE (acetylcholinesterase) is a serine hydrolase responsible for terminating neurotransmission by hydrolyzing the acetylcholine released on synaptic cleft. Studies of AChE as a target of pesticide toxicity have yielded several practical outcomes and are the basis for constructing biosensors. These devices are primarily designed to determine and quantify the inhibition of AChE by toxic chemicals.

Objective: to construct a biosensor based on acetylcholinesterase from the brain of the Prochilodus brevis fish, and to use the same as biomarker of agrochemicals that inhibit the enzyme.

Methods: Acetylcholinesterase was isolated from curimatã fish brain (prochilodus brevis) and partially purified using ammonium sulfate precipitation followed by size-exclusion chromatography (ChE1). AChE from curimatã fish brain was directly immobilizes on the surface of glassy carbon electrode modified with multi-walled carbon nanotubes.

Results: Acetylcholinesterase was characterized as having a specific activity of 0.194U/mg. The optimum activity was found at pH 8.5, phosphate buffer 0.7µM, 28ºC and exhibited a thermostability at 37°C. The glassy carbon modified electrode exhibits excellent electrocatalytic activity to the increase of thiocholine, with a linear response in the 0.05 mM to 0.85 mM concentration range, with a 73µM limit of detection and with a 240µM limit of quantification.

Conclusion: The AChE from curimatã fish brain allowed the modification of the glassy carbon electrode providing a potential sensor detection system that can be used for determination of thiocholine (pesticides) in real samples of environmental importance.

Introduction

Acetylcholinesterase (AChE) is present in all vertebrates, particularly in the muscles and nervous tissues [1]. AChE is a serine hydrolase (EC. 3.1.1.7) localized between the nerve terminal and post-synaptic membrane responsible for terminating neurotransmission by hydrolyzing the acetylcholine released on synaptic cleft, being one of the most efficient enzyme reactions [1,2]. Studies of AChE as a target of pesticide toxicity have yielded several practical outcomes and are the basis for constructing biosensors. These devices are primarily designed to determine and quantify the inhibition of AChE by toxic chemicals. Depending on the extraction source, these enzymes can have different substrate specificity and susceptibility to inhibitors [3]. The several different molecular forms which are dependent of enzyme gene shown variable catalytic properties of AChE [4,5]. Apart these, the physiological meaning of different forms and molecular interactions allow various types of AChE in different tissues and physiological conditions [4]. These various AChE forms have been a motivation for build a voltammetry biosensor applied for a specific ecosystem.

Fish are a group of great importance in environmental toxicity evaluations, as well as being present in various environments and present wide geographical distribution, still participate in different trophic levels of the food chain, being regarded as excellent biological models to study. Prochilodus brevis is a native fish of the San Francisco river; one of the largest watercourses of Brazil, has a huge regional importance of viewpoints ecological, economic and social. Besides, the effluents generated by the disorderly occupation are contamination promoted using agrochemicals of the agricultural activity in the surroundings of the river, which generates a concern about the contamination of the aquatic systems. The present work proposes to construct a biosensor based on acetylcholinesterase from the brain of the Prochilodus Brevis fish, native to the region, and to use the same as biomarker of agrochemicals that inhibit the enzyme, providing greater specificity. The electrochemical system builded will provide a biosensor for sensing thiocholine from AChE isolated of a real biological model.
Materials and Methods

Partial Purification of Prochilodus Brevis AChE

Prochilodus brevis brains were captured and collected in fish farming of San Francisco hydroelectric company (CHESF) in the Bahia state, Brazil. The brains were extracted and stored in tubes immediately immersed in liquid nitrogen and then frozen in the freezer until preparation of the crude extract. Brains were macerated with suspension buffer (20mM Tris-HCl, pH 7.5 in 0.1% Triton) followed by homogenization in glass homogenizer. The crude extract was subjected to 5 cycles on ultrasonic device for 15 seconds each cycle at 15 second intervals in an ice bath. For 20 minutes at 4°C the material was centrifuged at 10,000 rpm. The supernatant (cell free extract) was collected and stored at 4°C until the salt precipitation step. The salting out (salt precipitation) method was applied with ammonium sulphate on the cell free extract. The highest AChE activity was found on 40% (w/v) fraction after 3h shaking. The fraction was dialyzed three times and keeping overnight to remove salt excess. The enzymatic sample was applied directly to a Sephacryl S-100 (60 cm x 1 cm) equilibrated with Tris-HCl 20mM in Triton x100 (0.1%), NaCl 0.25M, pH 8.0. Elution of the fractions containing AChE was eluted with the same buffer. The fraction having was concentrated by poly (ethylene glycol) (PEG) on membrane filter. Finally, the concentrate fraction containing highest AChE activity (ChE1) were stored at 4°C awaiting the electrochemical measurements [6].

Enzyme Assay

AChE activity was measured by colorimetric method of [7]: 93 mM of potassium phosphate buffer, pH 8,0; 100µL enzyme sample; 3.3mM of 5,5’-dithiobis (2-nitrobenzoic acid), DTNB; 0.5 mM of the substrate acetylthiocholine iodide (ATCh) and the absorbance variation (20 – 46 ºC) of buffer and enzyme sample incubated for 10 min for each temperature show optimum temperature and then, stability of AChE from Prochilodus brevis was characterized by denaturation of ChE. At the temperature of 28ºC, immediately afterwards the sudden drop of activity from 31ºC to 46ºC is observed. Normally, the AChE show desestability with decrease activity. The optimum temperature (Figure 3A) of 28ºC, immediately afterwards the sudden drop of activity from 31ºC to 46ºC is observed. Normally, the AChE show desestability with decrease activity. The optimum temperature (Figure 3) showed highest activity at 0,08M, but above 1.0 M show desestability with decrease activity. The optimum temperature (Figure 2B) show highest activity at 0,08M, but above 1.0 M show desestability with decrease activity. The optimum temperature (Figure 2B) show highest activity at 0,08M, but above 1.0 M show desestability with decrease activity.

Results and Discussion

Partial Purification of Prochilodus Brevis AChE

The AChE cell free extract had specific activity of 0.310U/mg and then, salting out method increased the specific activity to 0.395U/mg. The further purification was carried out using size exclusion chromatography on a Sephacryl S-100 (Figure 1A), column equilibrated with Tris-HCl 20mM in Triton x100 (0,1%), NaCl 0.25M, pH 8.0. AChE was eluted using the same buffer and the chromatographic step decreased the specific activity to 0.194 U/mg.

Stability of AChE from Prochilodus brevis

The pH gradient affect enzyme stability by ionizations states of the side chains. The stability of AChE was tested by different pH at room temperature. The optimum pH (Figure 2A) was 8.5 with stability range at 8.0 to 9.5. The optimum ionic strength (Figure 2B) show highest activity at 0.08M, but above 1.0 M show desestability with decrease activity. The optimum temperature (Figure 3A) of 28ºC, immediately afterwards the sudden drop of activity from 31ºC to 46ºC is observed. Normally, the AChE show desestability with decrease activity. The optimum temperature (Figure 3) showed highest activity at 0,08M, but above 1.0 M show desestability with decrease activity.
the enzyme benefited the catalytic activity from 31 to 37ºC, after 37ºC the activity decrease of approximately 80% up to 46ºC was observed, suggesting structural instability of the enzyme at the level of an irreversible denaturation.

Figure 2: Effect of pH (A) and ionic strength (B) on activity of Prochilodus brevis AChE.

Figure 3: Optimum temperature (A) and thermal stability (B) of Prochilodus brevis AChE.

Electrochemical Studies

Initially were used CV of ferri/ferrocyanide for polishing the GCE. The MWCNT was adsorbed on the surface of GCE and CVs of ferri/ferrocyanide couple using modified and unmodified GCE for evaluate the barrier created after each modification step. The AChE samples were immobilized by adsorption on MWCNT surface. To study the electrocatalytic activity of AChE towards the oxidation of thiocholine was used the differential pulse voltammetry. (Figure 4) shows the analytical curve of current vs increase of ACTh concentration of 0.05 to 0.85 mM, for each fraction of the purification process. A linear response in the studied concentration range was obtained, whose limits of detection and quantification for each fraction (Table 1). The values were very close, where the ChE1 fraction with detection limit of 73μM and limit of quantification of 240 μM was higher. The purification process reflected in the oxidation potential, to favor the reduction of the potential between free cell fraction and ChE1.

Figure 4: Plot current vs increase of ATCh concentration of 0.05 to 0.85 mM, by DPV with modified electrode A) fraction cell-free, B) dialyzed e C) ChE1.
Table 1: Obtained data from the analytical curves for biosensor GCE/MWCNT/fraction of the brain extract of P. brevis.

| Fraction   | E / V | R   | DL / mM | QL / mM |
|------------|-------|------|---------|---------|
| Cell free  | 0.68  | 0.989| 0.075   | 0.26    |
| Dialyzed   | 0.70  | 0.985| 0.091   | 0.30    |
| ChE1       | 0.64  | 0.991| 0.073   | 0.24    |

The spherical shape of MWCNT have good biocompatibility with enzymes [11,12]. A special electron transfer between AChE and the electrode surface and adsorptive interaction helps to conserve the structure and catalytic properties of AChE, showing to be potentially useful for the construction of electrochemical biosensors. The combination of AChE interaction with MWCNT shows an increase in the surface area of the electrode, which is reflected in the current values obtained for the modified electrode.

Conclusion

As can be seen, the AChE from curimatã fish brain allowed the construction of the biosensor, both the cell free extract and the dialyzed and partially purified fraction, showed good cholinesterase enzymatic activity in the hydrolysis of acetylthiocholine monitored by the anodic oxidation of the generated thiocoline, with values of limits of detection and quantification close to each other. This work provides a potential sensor detection system that can be used for determination of thiocoline in real samples of environmental importance.

Acknowledgement

The authors are grateful to Brazilian agencies CNPq, CAPES, FAPEAL for financial support.

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ISSN: 2574-1241

DOI: 10.26717/BJSTR.2018.08.001655

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