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Research Article

Functional classification of esterases from leaves of *Aspidosperma polyneuron* M. Arg. (Apocynaceae)

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Abstract

Polyacrylamide gel electrophoresis system (PAGE) and inhibition tests for biochemical characterization of α- and β-esterases were used to obtain a functional classification of esterases from *Aspidosperma polyneuron*. The characterization of α- and β-esterases from young leaves of *A. polyneuron* by the PAGE system showed fourteen esterase isozymes. The differential staining pattern showed that Est-2 isozyme hydrolyzes β-naphthyl acetate; Est-6, Est-7 and Est-8 isozymes hydrolyze α-naphthyl acetate, and Est-1, Est-3, Est-4, Est-5, Est-9, Est-10, Est-11, Est-12, Est-13, and Est-14 isozymes hydrolyze both α- and β-naphthyl acetate. Inhibition pattern of α- and β-esterases showed that Folidol is a more potent inhibitor that Malathion, while Thiamethoxan (an insecticide with organophosphorus-like action) acts as an Est-4 and Est-6 inhibitor and induces the appearance of Est-5 and Est-7 isozymes as more intensely stained bands. Inhibition tests showed that OPC insecticides inhibit or activate plant esterases. Thus, plant esterases may be used as bioindicators to detect the presence and toxicity of residues of topically applied insecticides in agriculture and may be valuable for monitoring pollutants in the environment.

Key words: esterase classification, peroba-rosa, Ops, inhibition test.

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Introduction

The isozymes α- and β-esterases can be identified by electrophoresis due to their *in vitro* preferences for α- and β-naphthyl ester substrates (Holmes and Masters, 1967; Coates *et al.*, 1975). Furthermore, the most widely used functional classification of esterase activities relies primarily on their sensitivity to diagnostic concentrations of three groups of inhibitors, namely sulphydryl reagents (p-chloromercuribenzoate, pCMB), organophosphates (OPs), and eserine sulfate (Holmes and Masters, 1967). The use of these inhibitors distinguishes four classes of esterases: acetylesterases, which are not affected by any of the inhibitors and generally prefer aliphatic substrates involving acetic acid; arylesterases, which are only inhibited by the sulphydryl reagents and generally prefer aromatic substrates; carboxylesterases, which are only inhibited by the OPs and prefer aliphatic esters, generally of longer acids than acetic acid; cholinesterases, which are inhibited by both OPs and eserine sulfate and prefer charged substrates, such as cholinesters, to other aromatic or aliphatic esters.

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The inhibition tests for biochemical characterization of esterases from microbes (Lee *et al.*, 1987; Oakeshott *et al.*, 1993), vertebrates (Leibel, 1988; Higa *et al.*, 1989; Lith *et al.*, 1989; Deimiling and Wassmer, 1991) and insects, particularly species of *Drosophila* groups (Healy *et al.*, 1991; Lapenta *et al.*, 1995; 1998), have been important to decipher the different physiological processes in which esterases act. α- and β-esterases have been extensively studied in insects and are involved in different physiological processes, such as regulation of juvenile hormone levels (Kort and Granger, 1981), reproduction (Richmond *et al.*, 1980; Mane *et al.*, 1983), functioning of nervous system, and development of resistance to insecticides (Fournier *et al.*, 1993; Guillemaud *et al.*, 1997; Hemingway and Karunaratne, 1998). However, the functional characterization of plant esterases have been as yet scantly explored. The inhibition pattern of α- and β- esterases in plants showed arylesterase and a differential expression of an esterase isozyme in young unexpanded leaves of cassava plants as a marker of pathogenesis after infection with bacteria (Pereira *et al.* 2001).

In the present study, inhibition tests for the biochemical characterization of esterases by native polyacrylamide

gel electrophoresis were used to show its differential expression in leaves of *Aspidosperma polyneuron* after *in vitro* exposure to insecticides, and to obtain a functional classification of these esterases. The differential expression of esterases may be employed as a biosensor to detect the environmental pollution caused by insecticide compounds in plants.

Materials and Methods

In the state of Paraná, southern Brazil, the species *Aspidosperma polyneuron* M. Arg. (Apocynaceae) is known as "peroba-rosa". Samples of young leaves (8-10 mm long) of *A. polyneuron* were collected from native plants naturally grown for 54 years in the "Horto Florestal Dr. Luiz Teixeira Mendes", a 37-hectare Botanical Garden localized in the urban area of Maringá, northwestern region of Paraná (PR), Brazil. The "Horto Florestal" is a protected area for preservation of native and rare species which normally occur in small isolated or fragmented populations that have been reduced in size.

Electrophoretic analyses were carried out on samples consisting of individual young leaves collected from five 'peroba-rosa' tree. Four leaves of each tree were individually homogenized with a glass rod in an Eppendorf microcentrifuge tube using 100 μl of 1.0 M phosphate buffer, pH 7.0, containing 5% PVP-40, 0.01 M dithiothreitol (DTT), 10 mM sodium metabisulfite, 50 mM ascorbic acid, 1.0 mM EDTA, and 0.5% β-mercaptoethanol solution (Resende *et al.*, 2000). After homogenization, the samples were centrifuged at 25,000 rpm, for 30 min, at 4 °C in a Sorval 3K-30 centrifuge; supernatant (20 μL) was used for each sample. Each leaf was replicated 4 times in the same gel which, after electrophoresis, was vertically divided into 5 parts for the control and the four inhibition tests.

The polyacrylamide gels (12%) for vertical electrophoresis were prepared with 0.37 M Tris-HCl, and pH 8.8 as buffer (Ceron, 1988). The stack gel was prepared with 3.0 mL of acrylamide 10% and bis-acrylamide 0.5% dissolved in 3.0 mL of 0.24 M Tris-HCl, pH 6.8, 30 μL twice-distilled water, 250 μL ammonium persufate 2% and 30 μL TEMED. Gels were run during 3 h 30 min, at 25 °C, and constant 200 V. The running buffer was 0.1 M Tris-glycine, pH 8.3. Staining techniques by Johnson *et al.* (1966) and Steiner and Johnson (1973), modified by Ceron (1988), were used for esterase identification. The gels were soaked for 30 min in 50 mL 0.1 M sodium phosphate, pH 6.2, at room temperature. Esterase activity was visualized by placing the gels for 1 h in a staining solution prepared with 50 mL of sodium phosphate solution, 15 mg of β-naphthyl acetate, 20 mg of α-naphthyl acetate, 60 mg of Fast Blue RR salt, and 5 mL of N-propanol.

Inhibition tests for the biochemical characterization of esterases involved respectively 0.01 g (0.02 mM) p-chloromercuribenzoate (pCMB), 30 μL (1.6 mM) Malathion, 30 μL (51 mM) Folidol 500, or 0.5 mg (0.01 mM) Thiamethoxam 250 WG, each prepared with 100 mL twice-distilled water. These compounds were used separately in the pre-incubation and staining solutions of the different sections of each gel. Malathion and methyl-Parathion (the active substance of Folidol 500) are organophosphate pesticides extensively used to control a wide range of sucking and chewing pests in crops, fruits, and vegetables. Thiamethoxan insecticide is a new, second-generation product of a neonicotinoid compound of low toxicity used in crops to control sucking insects (Novartis Biociências S.A.); the neonicotinoid compound acts in a similar way as organophosphates. pCMB being an arylesterase inhibitor, while Malathion and Methyl-Parathion (OP compounds or OPCs) are carboxylesterase and cholinesterase inhibitors (Healy *et al.*, 1991).

Polyacrylamide gels were dried, as described by Ceron *et al.* (1992) and Lapenta *et al.* (1995). Gels were kept at room temperature and fixed for 1 h in a mixture of 7.5% acetic acid and 10% glycerol embedded in 5% gelatin, placed between two sheets of wet cellophane paper stretched on an embroidering hoop and left to dry for 24-48 h.

Results

Fourteen esterase isozymes were detected in leaves of the five analyzed plants of *A. polyneuron* when vertical polyacrylamide gel was used. The esterases were numbered in sequence starting from the anode, according to their decrease in negative charge (Figure 1A). Different esterase bands present characteristic colors, that is: α (black), β

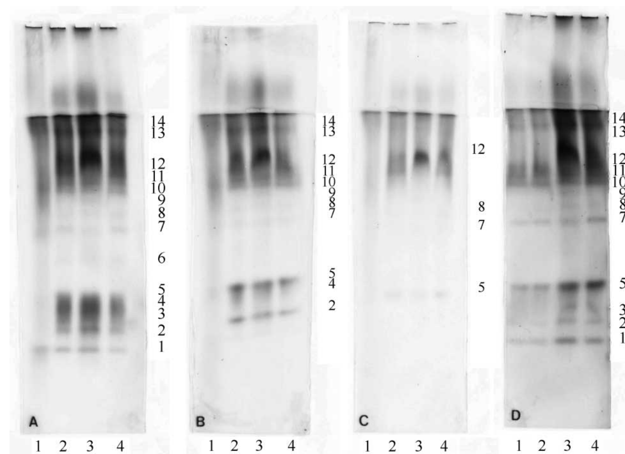

Figure 1 - Inhibition pattern for characterization of esterases from leaves of *Aspidosperma polyneuron* showing that Est-1, Est-3, Est-4, and Est-6 isozymes were inhibited in the presence of Malathion (B); Est-5, Est-7, Est-8, and Est-12 isozymes were detected as weakly stained bands by Folidol (C); Est-3, Est-4, and Est-6 isozymes were inhibited by Thiamethoxan (D). The gel in A shows α and β esterases in the absence of inhibitors. Lanes 1-4 correspond to leaf samples of different plants of *A. polyneuron*.

(red), and αβ (deep purple), since they hydrolyze different substrates, respectively: α-naphthyl acetate, β-naphthyl acetate, and both α- and β-naphthyl acetate. The staining specificity of each band was clearly determined by exposing gels to staining solutions containing only α-naphthyl acetate or/and ß-naphthyl acetate; they were: Est-2 (red), Est-6, Est-7 and Est-8 (black), and Est-1, Est-3, Est-4, Est-5, Est-9, Est-10, Est-11, Est-12, Est-13, and Est-14 (deep purple), (gels not shown).

The inhibition pattern of α-, β- and αβ-esterases from *A. polyneuron* showed that no esterase isozyme has been inhibited by pCMB (gels not shown). Est-1, Est-3 and Est-6 isozymes were inhibited by Malathion (Figure 1B). The bands of Est-5, Est-7, Est-8 and Est-12 isozymes presented weak staining (partial inhibition), while all other Est isozymes were inhibited by Folidol (Figure 1C). Est-4, and Est-6 isozymes were inhibited by insecticide Thiametoxan, whereas Est-5 and Est-7 isozymes presented the most intensely stained bands (Figure 1D).

Discussion

PAGE and inhibition tests for biochemical characterization of esterases from leaves of *A. polyneuron* showed one β-esterase (Est-2), three α-esterases (Est-6, Est-7 and Est-8), ten αβ-esterases (Est-1, Est-3, Est-4, Est-5, Est-9, Est-10, Est-11, Est-12, Est-13 and Est-14), and a selective sensitivity to organophosphate compounds for different esterases in these plants. Fourteen esterase isozymes have been detected by PAGE in leaves of different cassava cultivars (Pereira *et al.*, 2001). The number of esterases identified in the two plant species seems to be lower than the number of esterases detected in similar studies of mammalian and insect species (Oakeshott *et al.*, 1993; Lapenta *et al.*, 1998). Functional characterization of esterases from other species and from different tissues may be important to determine the actual number of different: α-, β-, and αβ-esterases in plants. Analyses of different tissues may reveal a larger number of esterases in plants. Esterase sensitivity to OPCs has been described in animals (Kaliste-Korhonen *et al.*, 1996; Cabello *et al.*, 2001), albeit their effect has not been explored in plants. Our results showed that Malathion and Methyl Parathion, used to control insect pests, were also able to inhibit α-, and αβ-esterases in leaves of *A. polyneuron*. These results are consistent with previously observations that significant similarities exist between most eukariotic carboxylesterases and all the eukaryotic cholinesterases sequenced to date (Oakeshott *et al.*, 1993; Mikhailov and Torrado, 2000). These enzymes are consequently placed in a single carboxyl/cholinesterase multigene family (Oakeshott *et al.*, 1999). It has been reported that the sequences required for the hydrolytic activity of carboxylesterases, acetylcholinesterases and cholesterol-esterases have been evolutionarily conserved to a high degree (Satoh and Hosokawa, 1998).

Cholinesterases or esterases with functional activity similar to cholinesterase have not been reported in plants. On the other hand, cholinesterases with non-cholinergic functions have been described in vertebrates. Such isozymes are termed nonspecific cholinesterases or pseudocholinesterases (Chatonnet and Lackridge, 1989). Thus, we propose the hypothesis that *A. polyneuron* esterases inhibited by Folidol represent examples of the plant's nonspecific cholinesterases that primarily have a non-cholinergic function in leaf tissues. It has been suggested that cholinesterases may have a proteolytic activity in addition to its cholinergic activity, acting as proteases, to regulate cell growth and development (Small, 1990). Carboxylesterase activity in plants has been correlated with the differentiation processes (Gahan *et al.*, 1983; Melati *et al.*, 1996) and inhibition of carboxylesterases was employed as a biosensor for detecting selenium compounds in *Thevetia peruviana* seeds (Saritha and Nanda Kumor, 2001).

We detected in *A. polyneuron* leaves a differential inhibition pattern for esterases by OPCs. Folidol acted as a more potent inhibitor than Malathion, while Thiamethoxan (an insecticide with an organophosphate-like action) acted as an Est-4 and Est-6 inhibitor and induced the enhancement of Est-5 and Est-7 bands staining intensities. This selective sensitivity of esterase to organophosphate compounds favors its binding to different OPCs. It has been proposed that, in the process of specific OPC detoxification, carboxylesterases participate by binding OPCs to their active center and sequestering them in the cell, or hydrolyzing the ester bonds of OPC molecules (Jakonovic *et al.*, 1996; Tang and Chambers, 1999). The not yet explained action of Thiamethoxan inducing the more intensely stained bands has also been detected in inhibition tests for certain species of insects (Ruvollo-Takasusuki *et al.* and Lapenta *et al.*, unpublished results).

The inhibition tests for biochemical and functional classification of esterases by PAGE in the current study were important since they differentiated the esterases of *A. polyneuron* leaves in α-, β-, and αβ-esterase, according to their substrate preferences, and showed that OPC insecticides were able to act by inhibiting or activating plant esterases. Therefore, it is possible that plant esterases may be used as bio-indicators to detect the presence and toxicity of insecticide residues topically applied in agriculture and may be valuable for monitoring pollutants in the environment. Manwell and Baker (1968) suggested that pollutants may influence biochemical polymorphism, since some enzymes (e.g. esterases) interact directly with pesticides and other pollutants. Detected differential expression of esterase activity in plant leaves after incubation with insecticide compounds indicates the importance of further investigations to research the effect of different types and concentrations of these compounds during field application to different plant species.

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