



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

Vanda Marilza de Carvalho, Rosimar Maria Marques, Ana Sílvia Lapenta
and Maria de Fátima P.S. Machado

*Departamento de Genética e Biologia Celular, Universidade Estadual de Maringá, Maringá,
Paraná, Brazil.*

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 than 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.

Received: June 26, 2002; accepted: March 26, 2003.

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 sulphhydryl reagents (p-chloromercuribenzoate, pCMB), organophosphates (OPs), and eserine sulfate (Holmes and Masters, 1967). The use of these inhibitors distinguishes four classes of esterases: acetyl esterases, which are not affected by any of the inhibitors and generally prefer aliphatic substrates involving acetic acid; arylesterases, which are only inhibited by the sulphhydryl 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.

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 scantily 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, north-western 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 micro-centrifuge tube using 100 μ L 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 μ L) 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 μ L twice-distilled water, 250 μ L ammonium persulfate 2% and 30 μ L 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 μ L (1.6 mM) Mala-

thion, 30 μ L (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

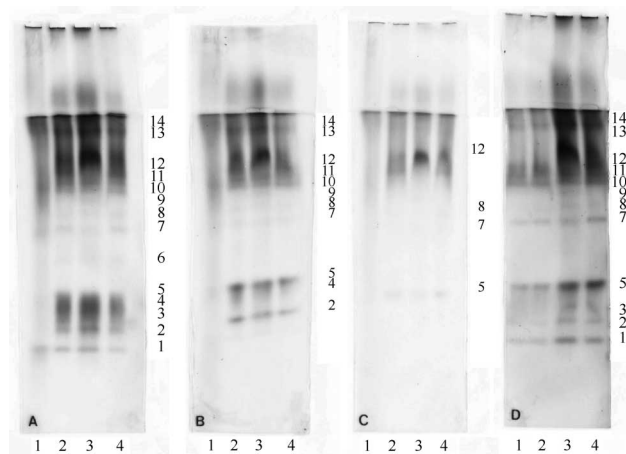


Figure 1 - Inhibition pattern for characterization of esterases from leaves of *Aspidosperma polyneuron* showing that Est-1, Est-3, Est-4, and Est-6 isozymes were inhibited in the presence of Malathion (B); Est-5, Est-7, Est-8, and Est-12 isozymes were detected as weakly stained bands by Folidol (C); 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 $\alpha\beta$ (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 β -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 $\alpha\beta$ -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 $\alpha\beta$ -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 $\alpha\beta$ -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 $\alpha\beta$ -esterases in leaves of *A. polyneuron*. These results are consistent with previously observations that significant similarities exist between most eukaryotic 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 (Sato 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 $\alpha\beta$ -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.

References

- Cabello G, Valenzuela M, Vilaxa A, Duran V, Rudolph I, Hrepic N and Calaf G (2001) A rat mammary tumor model induced by the organophosphorous pesticides parathion and malathion, possibly through acetylcholinesterase inhibition. *Environ Health Perspect* 109:471-479.
- Ceron CR (1988) Padrão de esterases no desenvolvimento de *Drosophila mulleri*, *D. arizonensis* e seus híbridos. Doctoral Thesis, Instituto de Biociências, Universidade de São Paulo, 142 p.
- Ceron CR, Santos JR and Campos Bicudo HEM (1992) The use of gelatin to dry cellophane wound slab gels in an embroidering hoop. *Brazil J Genet* 15:201-203.
- Coates PM, Mestriner MA., Hopkinson DA (1975) A preliminary genetic interpretation of the esterase isozymes of human tissues. *Ann Hum Genet* 39: 1-20.
- Chatonnet A and Lockridge O (1989) Comparison of butyrylcholinesterase and acetylcholinesterase. *Biochem J* 260:625-629.
- Deimilling O and Wassmer B (1991) Genetic characterization of esterase-28 (ES-28) of the house mouse. *Biochem Genet* 29:55-64.
- Fournier D, Mutero A, Pralavorio M and Bride JM (1993) *Drosophila* acetylcholinesterase mechanism of resistance to organophosphates. *Chem Biol Interactions* 87:233-238.
- Gahan PB, Rana MA and Phillips R (1983) Activation of carboxylesterases in root cortical parenchymal cells of *Pisum sativum* during xylem induction in vitro. *Cell Biochem Funct* 1:109-111.
- Guillemaud T, Makate N, Raymond M, Hirst B, and Callghan A (1997) Esterase gene amplification in *Culex pipiens*. *Insect Mol Biol* 6:319-327.
- Healy MJ, Dumancic MM and Oakeshott JG (1991) Biochemical and physiological studies of soluble esterases from *Drosophila melanogaster*. *Biochem Genet* 29:365-388.
- Hemingway J and Karunaratne SHPP (1998) Mosquito carboxylesterase: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. *Med Vet Entomol* 12:1-12.
- Higa HH, Manzi A and Varki A (1989) O-Acetylation and de-O-acetylation of sialic acids. Purification, characterization, and properties of a glycosylated rat liver esterase specific for 9-O-acetylated sialic acids. *Biol Chem* 264:435-442.
- Holmes RS and Masters CJ (1967) The developmental multiplicity and isoenzyme status of avian esterases. *Biochim Biophys Acta* 132:379-399.
- Johnson FM, Kanapi CG, Richardson RH, Wheeler MR and Stone WS (1966) An operational classification of *Drosophila* esterases for species comparison. *Univ Texas Publ* 6615:517-532.
- Jokanovic M, Kosanovic M and Maksimovic M (1996) Interaction of organophosphorus compounds with carboxylesterases in the rat. *Arch Toxicol* 13:261-268.
- Kaliste-Korhonen E, Tuovinen K and Hanninen O (1996) Interspecies differences in enzymes reacting with organophosphates and their inhibition by paraoxon in vitro. *Hum Exp Toxicol* 15:972-978.
- Kort CA and Granger NA (1981) Regulation of the juvenile hormone titer. *Ann Rev Entomol* 26:1-28.
- Lapenta AS, Campos Bicudo HEM, Ceron CR and Cordeiro JA (1995) Esterase patterns of species in the *Drosophila buzzatii* cluster. *Cytobios* 84:13-29.
- Lapenta AS, Campos Bicudo HEM, Ceron CR and Cordeiro JA (1998) Esterase patterns and phylogenetic relationships of species and strains included in the *Drosophila buzzatii* cluster. *Cytobios* 96:95-107.
- Lee H, To RJB and Latta RK (1987) Some properties of extracellular acetylxyylan esterase produced by the yeast *Rhodotorula mucilaginosa*. *App Environ Microbiol* 53:2831-2834.
- Leibel WS (1988) An analysis of esterase activities from surgeonfish tissue yields evidence of atypical pseudocholesterase. *Comp Biochem Physiol* 91B:437-447.
- Lith HA, Bieman M and Zutphen BFM (1989) Purification and molecular properties of rabbit liver esterase ES-1A. *Eur J Biochem* 184:545-551.
- Mane SD, Tompkins L and Richmond RC (1983) Male esterase-6 catalyzes the synthesis of a sex pheromone in *Drosophila melanogaster* females. *Science* 222:419-421.
- Manwell C and Baker CMA (1968) Genetic variation of isocitrate, malate and 6-phosphogluconate dehydrogenase in snails of the genus *Cepaea* - introgressive hybridization, polymorphism and pollution? *Comp Biochem Physiol* 26:195-209.
- Melati MR, Scialabba A and Bellani ML (1996) Carboxylesterase activity in the carrot seed and seedling. *Eur J Histochem* 40:159-166.
- Mikhailov AT and Torrado M (2000) Carboxylesterases moonlight in the male reproductive tract: a functional shift pivotal for male fertility. *Front Bioscience* 5:53-62.
- Oakeshott JG, Claudianos C, Russel RJ and Robin GC (1999) Carboxyl/cholinesterases: a case study of the evolution of a successful multigene family. *BioEssays* 21:1031-1042.
- Oakeshott JG, van Papenrecht EA, Boyce TM, Healy MJ and Russell RJ (1993) Evolutionary genetics of *Drosophila* esterases. *Genetics* 90:239-268.
- Pereira AJ, Lapenta AS, Vidigal-Filho PS and Machado MFPS (2001) Differential esterase expression in leaves of *Manihot esculenta* Crantz infected by *Xanthomonas axonopodis* pv. *manihotis*. *Biochem Genet* 39:289-296.
- Resende AG, Vidigal-Filho PS and Machado MFPS (2000) Isozyme diversity in cassava cultivars (*Manihot esculenta* Crantz). *Biochem Genet* 38:203-215.
- Richmond RC, Gilbert DG, Sheehan KB, Gromko MH and Butterworth FM (1980) Esterase 6 and reproduction in *Drosophila melanogaster*. *Science* 207:1483-1485.
- Saritha K and Nanda Kumar NV (2001) Qualitative detection of selenium in fortified soil and water samples by a paper chromatographic-carboxyl esterase enzyme inhibition technique. *J Chromatogr A* 919:223-228.
- Satoh T and Hosokawa M (1998) The mammalian carboxylesterases: from molecules to functions. *Ann Rev Pharmacol Toxicol* 38:257-288.
- Small DH (1990) Non-cholinergic actions of acetylcholinesterases: proteases regulating cell growth and development? *Trends Biochem Sci* 15:213-216.
- Steiner WWM and Johnson WE (1973) Techniques for electrophoresis of Hawaiian *Drosophila* US - IBP. *Island Ecosyst. Tech Rep* 30:1-21.
- Tang J and Chambers JE (1999) Detoxification of paraoxon by rat liver homogenate and serum carboxylesterases and A-esterases. *J Biochem Mol Toxicol* 13:261-268.