Tryptophan fluorescence quenching as a binding assay to monitor protein conformation changes in the membrane of intact mitochondria

S. MD. Akbar[1,2] · K. Sreeramulu[2] · Hari C. Sharma[1]

Abstract Intrinsic protein fluorescence is due to aromatic amino acids, mainly tryptophan, which can be selectively measured by exciting at 295 nm. Changes in emission spectra of tryptophan are due to the protein conformational transitions, subunit association, ligand binding or denaturation, which affect the local environment surrounding the indole ring. In this study, tryptophan fluorescence was monitored in intact mitochondria at 333 nm following excitation at 295 nm in presence of insecticides using spectrofluorometer. Methyl-parathion, carbofuran, and endosulfan induced Trp fluorescence quenching and release of cytochrome c when incubated with the mitochondria, except fenvalarate. Mechanism of insecticide-induced mitochondrial toxicity for the tested insecticides has been discussed. Reduction in the intensity of tryptophan emission spectra of mitochondrial membrane proteins in presence of an increasing concentration of a ligand can be used to study the interaction of insecticides/drugs with the intact mitochondria. Furthermore, this assay can be readily adapted for studying protein–ligand interactions in intact mitochondria and in other cell organelles extending its implications for pesticide and pharma industry and in drug discovery.

Keywords Mitochondria · Trp fluorescence quenching · Cytochrome c · Insecticide

✉ Hari C. Sharma
  h.sharma@cgiar.org

[1] Entomology, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana 502324, India

[2] Department of Biochemistry, Gulbarga University, Gulbarga, Karnataka 585106, India

Introduction

Fluorescence spectroscopy has proved to be useful in studies on ligand binding. Steady-state fluorescence quenching and fluorescence polarization are the primary techniques for studying structure and function of proteins. In fluorescence spectroscopy, light absorption promotes an electron to an excited state, resulting in light emission when the excited electron returns to the ground state undergoing a radiative transition. Since some of the light energy initially absorbed is lost in transition between vibrational energy levels (internal conversion), the emitted light energy is always of a longer wavelength (Moller and Denicola 2002). Fluorescence quenching refers to the process that decreases the fluorescence intensity of a sample. Proteins display fluorescence, and the intrinsic protein fluorescence is due to aromatic amino acids, mainly tryptophan, considering that phenylalanine has a very low quantum yield, and emission by tyrosine in native proteins is often quenched. Fluorescence is mainly due to tryptophan, which can be selectively measured by exciting at 295 nm, because there is no absorption by tyrosine at this wavelength. Tryptophan fluorescence is highly sensitive to the environment polarity, and shifts in its emission spectrum towards lower wavelengths (blue shift) can be observed as the increased hydrophobicity. Changes in emission spectra from tryptophan can be seen in response to protein conformational transitions, subunit association, ligand binding, or denaturation, which affect the environment surrounding the indole ring. The lifetime of a fluorophore (i.e. the time the fluorophore spends in the excited state) is very short, ranging from 0.5 to 100 ns, but sufficiently long for the excited fluorophore to react with external molecules to yield an excited product that decays by a non-radiative pathway, and therefore, diminishes the fluorescence quantum yield. This process is known as dynamic or collisional

quenching, and the external molecule is known as the quencher (Lakowicz 1999). Many biological molecules display fluorescence, such as reduced nicotinamide dinucleotide (NADH), oxidized flavins (FAD, adenine dinucleotide, FMN, and mononucleotide), pyridoxal phosphate, chlorophyll, and proteins (Lakowicz 1999).

In addition, the quenching reaction can be used not only to probe topological features of the macromolecular structures, but also to follow protein conformation changes that affect accessibility to tryptophan. Any treatment of the native protein that involves a change in the tryptophan environment can be followed by fluorescence quenching. The tryptophan residue acts as a reporter group for local conformation change. Several studies have used Trp fluorescence quenching to study interaction of purified proteins with drugs (Aurade et al. 2010a, 2010b; Aurade et al. 2012; Ahmad et al. 2015; Abdelhameed et al. 2015). Static quenching of anti-tuberculosis drugs with Aβ-42 peptide and hen egg white lysozyme, anticancer drug with human serum albumin and galactose with soybean agglutinin was studied using Trp fluorescence (Chaturvedi et al. 2015a; Alam et al. 2015a, b). Interaction of taurine with human serum albumin and surfactants with lysozyme and *Rhizopus niveus* lipase protein was studied using fluorescence spectroscopy (Chaturvedi et al. 2015b, c; Alam et al. 2015c).

Insecticides are neurotoxic to insects, and there are a number of reports asserting their harmful effects on insects as well as on the non-target organisms affecting mitochondrial bioenergetics. Pesticides adversely affect mitochondrial metabolism in fish (Reddy and Phillip 1992) and mammals (Gassner et al. 1997). Organophosphates impair mitochondrial energy metabolism, resulting in oxidative stress and neuronal apoptosis in rat brain (Kaur et al. 2007). Methyl-parathion results in deleterious effects on oxidative phosphorylation and membrane depolarization in rat liver mitochondria under in vitro conditions (Moreno and Madeira 1990), while carbofuran impairs mitochondrial function in the rat brain (Kamboj et al. 2008). Toxicity of chlorpropham - a carbamate insecticide is associated with the rapid depletion of ATP via impairment of mitochondrial function (Nakagawa et al. 2004). Carbaryl inhibits mitochondrial bioenergetics and succinate dehydrogenase in rats (Morena et al. 2007), while chlorfenvinphos induces oxidative stress in rat liver mitochondria (Lukaszewicz-Hussain and Moniuszko-Jakoniuk 2004). The present studies were undertaken to study the interaction of insecticides with the intact mitochondria isolated from a polyphagous pest, cotton bollworm, *Helicoverpa armigera* (Hubner), an important constraint to crop production by using binding assay based on tryptophan fluorescence.

Materials and methods

Materials

Bovine serum albumin (BSA) were purchased from Sigma Aldrich (Mumbai, India), sucrose Qualigens (Mumbai, India), methyl-parathion (99.3 %) and carbofuran (99 %) from Pesticide Analysis Laboratory, Gulbarga, and endosulfan (99 %) and fenvalerate (99.4 %) from Dow Agro Sciences (gifted from Dr. Sharom, Department of Environmental Biology, University of Guelph, Canada). All the other chemicals used were of analytical grade.

Insects

The cotton bollworm, *H. armigera* larvae were obtained from the insect rearing laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana, India. The larvae were reared on a chickpea based semi-synthetic diet under laboratory conditions at 27 ± 1 °C, 65 ± 5 % RH, and 12 h photoperiod (Chitti Babu et al. 2014).

Isolation of mitochondria

The fourth-instar *H. armigera* larvae were starved for 3 h, their midgut content removed, washed in cold distilled water, and then homogenized in Dounce homogenizer under cold conditions in the isolation media (0.25 M sucrose solution containing 0.1 % defatted BSA). The homogenate was filtered through a moist muslin cloth, and the filtrate centrifuged at 800×g for 10 min at 4 °C. The residue was re-suspended in extraction buffer, and centrifuged at 800×g for 5 min. The supernatants from both the centrifugations were combined and centrifuged at 8000×g for 10 min. The mitochondrial pellet was re-suspended in the 0.25 M sucrose solution and kept in ice until used (Akbar et al. 2012). Protein concentration was determined by Lowry's method using BSA as standard (Lowry et al. 1951).

Fluorescence spectroscopy

Steady-state fluorescence measurements were performed with Cary Varian Eclipse fluorescence spectrophotometer (les Ulis, France), using a 10-mm path length quartz cell with a Teflon stopper. Both excitation and emission slit widths were 5 nm. The affinity of binding of insecticides such as methyl-parathion, carbofuran, endosulfan and fenvalarate with the membrane proteins of mitochondria was determined by Trp fluorescence quenching titrations. Intact mitochondria (200 mg protein) was titrated in 50 mM phosphate buffer, pH 7.4, with increasing concentrations of insecticides, while quenching of Trp fluorescence was monitored at 333 nm following excitation at 295 nm (slit width for both was 5 nm). One insecticide from each class was selected to study their interaction with the

mitochondrial membrane proteins. Dissociation constant (K_d) and maximum fluorescence (ΔF_{max}) values were computed following fitting of the data to an equation describing binding to a single affinity site.

$$\frac{\Delta F/F_0 \times 100 = (\Delta F/F_{max} \times 100) \times [S]}{K_d + [S]}$$

Where ($\Delta F/F_0 \times 100$) represents the percent change in fluorescence intensity relative to the initial value after addition of insecticide at a concentration [S], and $\Delta F_{max}/F_0 \times 100$ is the maximum percent quenching of the fluorescence intensity that occurs upon saturation of the substrate-binding site.

Trp fluorescence quenching of intact mitochondria by acrylamide

Intact mitochondria (200 mg protein) were titrated in 50 mM phosphate buffer, pH 7.4, with increasing concentrations of acrylamide (0–50 μM). Fluorescence emission was measured at 330 nm following excitation at 295 nm (bandwidths of 5 nm). Quenching data were analyzed using the Stern–Volmer equation,

$$F_0/F = 1 + K_{SV}[Q]$$

where F_0 and F are the fluorescence intensities in the absence and presence of acrylamide, respectively, [Q] is the acrylamide concentration, and K_{SV} is the Stern–Volmer quenching constant. For a collisional quenching mechanism, a plot of F_0/F versus [Q] gives a line with a slope of K_{SV}.

Release of cytochrome c

Mitochondria (1 mg protein) isolated from fourth-instar larvae of *H. armigera* were incubated in 50 mM phosphate buffer, pH 7.2, containing 50 mM of insecticide (methyl-parathion, carbofuran, endosulfan and fenvalarate) for 1 h in the presence of 0.1 mM Ca^{2+}. The reaction mixtures were centrifuged at 12,000×g for 10 min at 4 °C to obtain the mitochondrial pellet. The supernatants were further spun at 12,000×g for 1 h to eliminate mitochondrial fragments. The supernatants were collected, and the released cytochrome c was estimated according to Chamberlin (2004).

Results and discussion

Proteins in biological membranes represent up to 30 % of all proteins. The mitochondrial outer membrane consists of 40 % lipids and 60 % proteins. Among the proteins it includes a diverse set of enzymes, components of precursor protein translocation and insertion machineries, pore forming proteins and proteins mediating mitochondrial fusion and fission (Walther and Rapaport 2009). Certain membrane proteins are functional as monomers while others assemble into oligomeric structures spanning across the membrane to perform their biochemical role. Due to complexity and dynamics of their arrangement, they are hardly traceable, and it is nearly impossible to study their interaction with any ligand in a lipid bilayer model. In this context, tryptophan fluorescence provides a valuable tool in studying interaction of membrane proteins with ligand molecules. Tryptophan residues being positioned at the vicinity of the core of the protein, Trp fluorescence spectrum can be used as a local conformational change at the core of the protein. Emission spectra of the intact mitochondria when excited at 295 nm revealed the presence of two shoulders of λ_{max} 333 and 404 nm with a valley at 370 nm (Fig. 1). Addition of insecticide to the isolated mitochondria resulted in quenching of the fluorescence at both the shoulders. Quenching of Trp fluorescence emission due to insecticides was measured as reduction of fluorescence intensity at λ_{max} 333 nm, which suggest the formation of an association between the macromolecule and the insecticides due to change in Trp environment. Decrease in fluorescence intensity with a slight red shift at λ_{max} 333 nm is due to the exposure of Trp residues to polar environment. The red and blue shift indicates the decrease or increase in hydrophobicity around Tyr and Trp residues, respectively (Chen et al. 2014). Methyl-parathion, carbofuran and endosulfan displayed substantial levels of Trp quenching except fenvalerate. Analysis of experimental data showed that all the insecticides except fenvalerate interacted with the mitochondrial membrane proteins with relatively high affinity with K_d (dissociation constant) values of 16, 28, 32 μM, and K_{sv} (Stern-Volmer constant) values of 0.51×10^4, 0.94×10^4, and 6.2×10^4 M^{-1}, for methyl-parathion, carbofuran, and endosulfan, respectively. Fenvalarate - a synthetic pyrethroid, with a different mode of action, did not showed any change in the fluorescence when added to the mitochondrial extract indicates that the insecticide have no interaction with the mitochondrial membrane proteins. The Trp residues that absorb near-UV radiation receive the additional energy equal to that of one photon, and become electronically excited. Upon return to the ground state, the molecules dissipate the energy by breaking chemical bonds or by re-radiating photons at another frequency. Covalent damage of Trp residues can lead to cleavage of the indole ring, possibly due to formation of a short-lived Trp cation (Bent and Hayon 1975; Bryant et al. 1975). Therefore, binding of insecticides brings out changes in the environment of tryptophan residues in the intact mitochondria as indicated by the shifts in λ_{max}. Quenching due to acrylamide is considered as an indicator of the accessibility of protein Trp residues to solvent under various conditions. The linear nature of the Stern-Volmer plot (Stern-Volmer constant, $K_{SV} = 1 \times 10^4$ M^{-1}) of F_0/F versus acrylamide concentration was obtained when Trp fluorescence of intact mitochondria was measured in the presence of
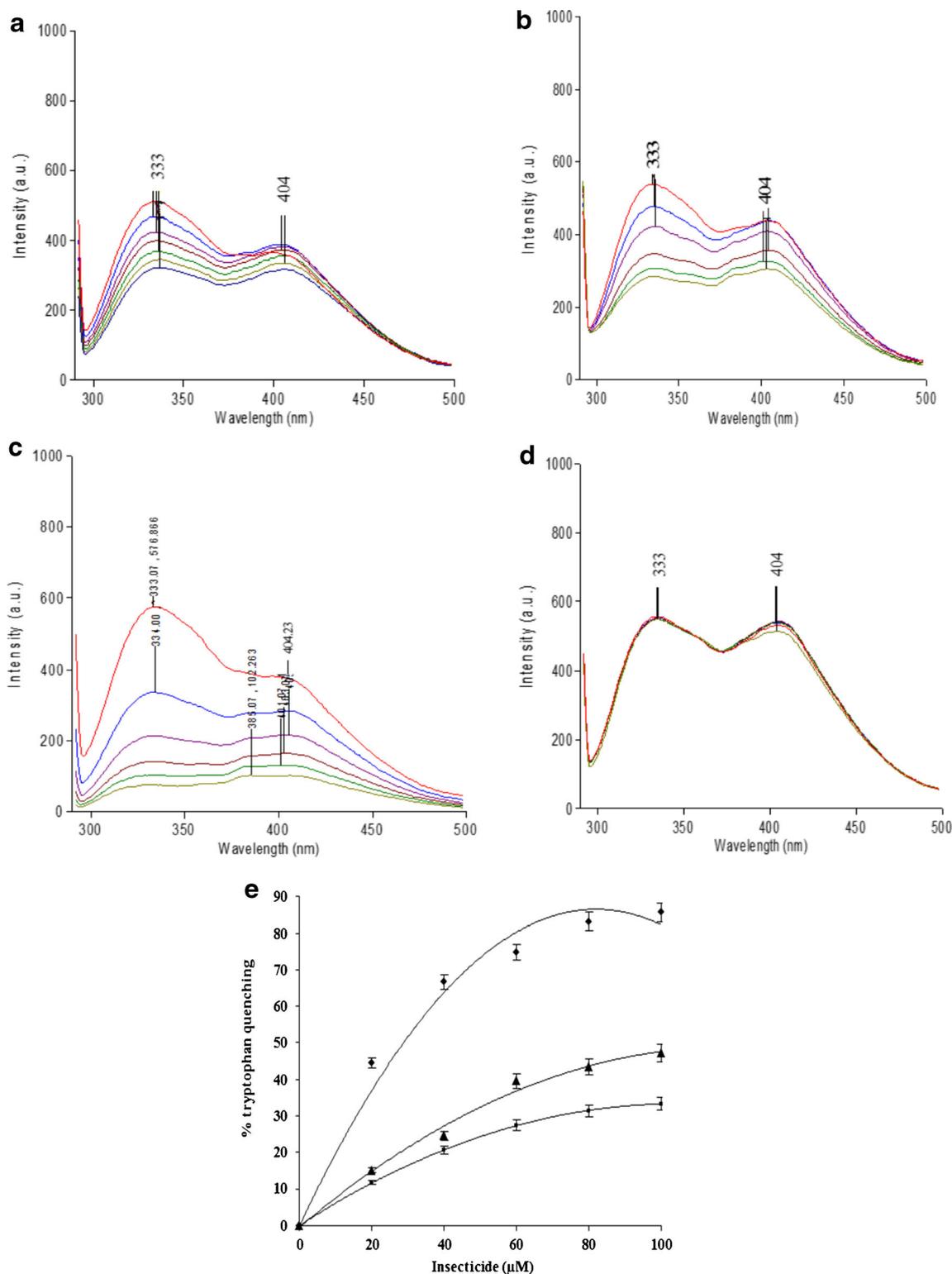
Fig. 1 Fluorescence emission spectra of mitochondrial membrane proteins in the absence of insecticide (*red line*) and in presence of increasing concentrations of insecticides (0–100 μM) (from red line onwards), methyl-parathion **a**, carbofuran **b**, endosulfan **c** and fenvalarate **d**. **e** Binding of methyl-parathion (♦), carbofuran (▲) and endosulfan (■) with the membrane proteins of intact mitochondria isolated from *H. armigera* as assessed by tryptophan fluorescence quenching. The data represent mean ± SD (*n* = 3)

acrylamide (Fig. 2), suggests the presence of one type of Trp residues, with all of them being equally accessible to the quencher, as previously reported for mammalian Pgp (Liu et al. 2000).
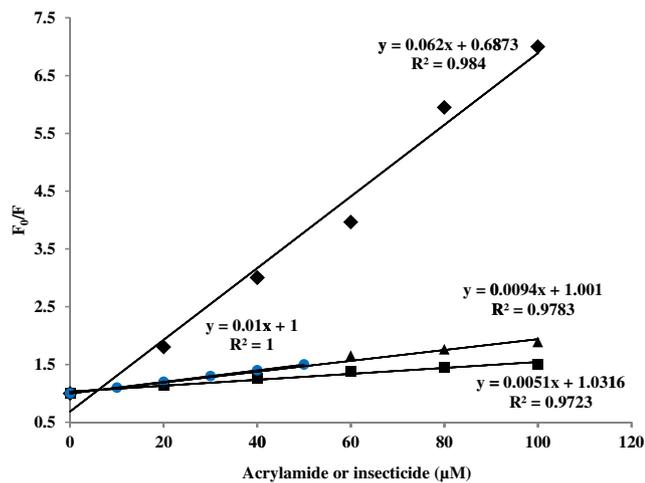
Fig 2 Trp fluorescence quenching of intact mitochondria in presence of insecticide and acrylamide. Trp fluorescence of intact mitochondria was assessed in presence of varying concentration of methyl-parathion (■), carbofuran (▲), endosulfan (♦) and acrylamide (●). The data represent the means ± SD of three independent experiments

Fig. 3 Release of cytochrome c. Cytochrome c release was estimated by incubating mitochondria (1 mg protein) isolated from *H. armigera* larvae in the presence of methyl-parathion, carbofuran, endosulfan and fenvalarate. Control mitochondria were incubated with the phosphate buffer, pH 7.0. The data represent mean ± SD ($n = 3$). Data were subjected to analysis of variance (ANOVA). F-test was used to judge the significance of differences between the treatments, while the least significant difference (LSD) was used to test significance of differences between the treatment means at $p \leq 0.05$

Insecticides induce apoptosis or programmed cell death both in vitro and in vivo (Wu et al. 2003; Wu and Liu 2000; Hossain and Richardson 2011). Apoptosis is executed by a subfamily of cysteine proteases known as caspases. A major caspase activation pathway is the cytochrome c-initiated pathway. In this pathway, apoptotic stimuli cause cytochrome c release from mitochondria, which in turn induces a series of biochemical reactions that result in caspase activation and subsequent cell death. In the present study, methyl-parathion, carbofuran and endosulfan induced the efflux of cytochrome c when incubated with isolated mitochondria from *H. armigera* larvae in the presence of Ca^{2+}. Cytochrome c levels in the supernatant were found to be 1.76, 1.56 and 1.35 nmol/mg protein for methyl-parathion, carbofuran and endosulfan, respectively, whereas mitochondria incubated with fenvalarate did not induce the release of cytochrome c (Fig. 3). The results indicated that all the insecticides, except fenvalarate, induced mitochondrial toxicity, among the tested insecticides. Earlier studies have shown that organophosphates (Kashyap et al. 2010), carbamates (Li et al. 2015) and organochlorine (Kitazawa et al. 2003) insecticides induce apoptosis through mitochondrial-dependent pathway, and similar indications were obtained in the present study. *In vivo* exposure of a synthetic pyrethroid - deltamethrin, have been shown to reduce mitochondrial cytochrome c levels (Chen et al. 2007), and induce apoptosis through mitochondrial-independent pathway. Deltamethrin-induced apoptosis is initiated through a mitochondrial-independent pathway due to its interaction with Na^{+} channels, leading to calcium overload and activation of the endoplasmic reticulum (ER) stress pathway (Hossain and Richardson 2011). Differences in the ability of the insecticides in inducing Trp
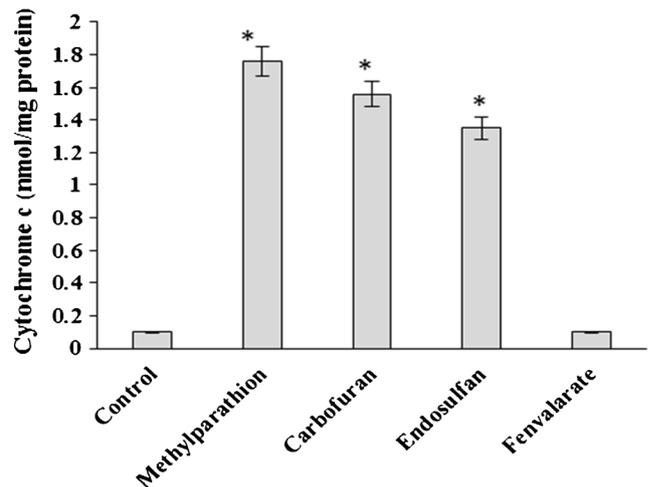
fluorescence quenching and efflux of cytochrome c in the intact mitochondria may be attributed to the specific functional groups possessed by the insecticides which interact with the components of cell organelles.

For quenching to occur, the fluorophore and quencher must be in contact with each other. The Trp quenching observed with the insecticides, except fenvalarate, were due to the interaction of insecticides with the Trp residues of integral proteins protruding out from the lipid membrane in the cell organelle, which brings conformational changes in the proteins studded in the mitochondrial membrane thereby inducing the release of cytochrome c and further leading to caspase activation and subsequent cell death. The tryptophan fluorescence quenching assay is a direct and clear demonstration of interaction of insecticides with the mitochondrial membranes proteins and the role of insecticides in inducing mitochondrial toxicity. This technique will be useful in investigating the toxicity of various drugs or chemicals on mitochondria in different organisms.

Conclusions

Results showed that the insecticides, methyl-parathion, carbofuran, and endosulfan quenched Trp fluorescence in the mitochondria of *H. armigera* indicate that the insecticides induced mitochondrial toxicity through interaction with the membrane proteins. Interaction of pesticide/drug molecules with the mitochondrial

membrane proteins can be easily studied by tryptophan quenching assay in intact mitochondria. This assay will be useful for understanding the mode of action, toxicity and biosafety of many chemicals to the target and non-target organisms, especially in pesticide and pharma industry and in drug discovery.

References

Abdelhameed AS, Alam P, Khan RH (2015) Binding of janus kinase inhibitor tofacitinib with human serum albumin: multi-technique approach. J Biomol Struct Dyn. doi:10.1080/07391102.2015.1104522

Ahmad B, Muteeb G, Alam P, Varshney A, Zaidi N, Ishtikhar M, Badr G, Mahmoud MH, Khan RH (2015) Thermal induced unfolding of human serum albumin isomers: assigning residual alpha helices to domain II. Int J Biol Macromol. doi:10.1016/j.ijbiomac.2015.02.003

Akbar SMD, Sharma HC, Jayalakshmi SK, Sreeramulu K (2012) Interaction of plant cell signaling molecules, salicylic acid and jasmonic acid, with the mitochondria of Helicoverpa armigera. J Bioenerg Biomembr 44:233–241

Alam P, Naseem F, Abdelhameed AS, Khan RH (2015a) Effect of galactose on acid induced molten globule state of soybean agglutinin: biophysical approach. J Mol Struct 1099:149–153

Alam P, Chaturvedi SK, Anwar T, Siddiqi MK, Ajmal MR, Badr G, Mahmoud MH, Khan RH (2015b) Biophysical and molecular docking insight into the interaction of cytosine β-D arabinofuranoside with human serum albumin. J Lumin 164:123–130

Alam P, Rabbani G, Badr G, Badr BM, Khan RH (2015c) The surfactant-induced conformational and activity alterations in Rhizopus niveus lipase. Cell Biochem Biophys 71:1199–1206

Aurade RM, Jayalakshmi SK, Sreeramulu K (2010a) P-glycoprotein ATPase from the resistant pest, Helicoverpa armigera: purification, characterization and effect of various insecticides on its transport function. Biochim Biophys Acta 1798:1135–1143

Aurade RM, Jayalakshmi SK, Sreeramulu K (2010b) Modulatory effects of natural curcuminoids on P-glycoprotein ATPase of insecticide-resistant pest Helicoverpa armigera (Lepidopetera: noctuidae). J Membrane Biol 236:271–278

Aurade RM, Jayalakshmi SK, Udikeri SS, Sreeramulu K (2012) Modulation of P-glycoprotein ATPase of Helicoverpa armigera by cholesterol: effects on ATPase activity and interaction of insecticides. Arch Insect Biochem Physiol 79:47–60.

Bent DV, Hayon E (1975) Excited state chemistry of aromatic amino acids and related peptides. III Tryptophan J Am Chem Soc 97:2612–2619

Bryant FD, Santus R, Grossweiner LI (1975) Laser flash-photolysis of aqueous tryptophan. J Phys Chem 79:2711–2716

Chamberlin ME (2004) Control of oxidative phosphorylation during insect metamorphosis. Am J Physiol Regul Integr Comp Physiol 287:314–321

Chaturvedi SK, Zaidi N, Alam P, Khan JM, Qadeer A, Siddique IA, et al. (2015a) Unraveling comparative anti-amyloidogenic behavior of pyrazinamide and D-cycloserine: a mechanistic biophysical insight. PLoS one 10(8):e0136528. doi:10.1371/journal.pone.0136528

Chaturvedi SK, Alam P, Khan JM, Siddique MK, P Kalaiarasan, N Subbarao, Z Ahmad, R.H. Khan. (2015b), Biophysical insight into the anti-amyloidogenic behavior of taurine. Int J Biol Macromol doi:10.1016/j.ijbiomac.2015.06.035.

Chaturvedi SK, Khan JM, Siddiqui MK, Alam P, Khan RH (2015c) Comparative insight into surfactants mediated amyloidogenesis of lysozyme. Int J Biol Macromol 83:315–325

Chen D, Huang X, Liu L, Shi N (2007) Deltamethrin induces mitochondrial membrane permeability and altered expression of cytochrome C in rat brain. J Appl Toxicol 27:368–372

Chen Z, Xu H, Zhu Y, Liu J, Wang K, Wang P, et al. (2014) Understanding the fate of an anesthetic, nalorphine upon interaction with human serum albumin: a photophysical and mass-spectroscopy approach. RSC Adv 4:25410–25419

Chitti Babu G, Sharma HC, Madhumati T, Raghavaiah G, Murthy KVMK, Rao VS (2014) A semi-synthetic chickpea flour based diet for long-term maintenance of laboratory culture of Helicoverpa armigera. Indian J Entomol 76:336–340

Gassner B, Wüthrich A, Sholtysik G, Solioz M (1997) The pyrethroids permethrin and cyhalothrin are potent inhibitors of the mitochondrial complex I. J Pharmacol Exp Therapeut 281:855–860

Hossain MM, Richardson JR (2011) Mechanism of pyrethroid pesticide–induced apoptosis: role of calpain and the ER stress pathway. Toxicol Sci 122(2):512–525

Kamboj SS, Kumar V, Kamboj A, Sandhir R (2008) Mitochondrial oxidative stress and dysfunction in rat brain induced by carbofuran exposure. Cell Mol Neurobiol 28:961–969

Kashyap MP, Singh AK, Siddiqui MA, Kumar V, Tripathi VK, Khanna VK, Yadav S, Jain SK, Pant AB (2010) Caspase cascade regulated mitochondria mediated apoptosis in monocrotophos exposed PC12 cells. Chem Res Toxicol 23:1663–1672

Kaur P, Radotra B, Minz RW, Gill KD (2007) Impaired mitochondrial energy metabolism and neuronal apoptotic cell death after chronic dichlorvos (OP) exposure in rat brain. Neurotoxicology 28(6):1208–1219

Kitazawa M, Anantharam V, Kanthasamy AG (2003) Dieldrin induces apoptosis by promoting caspase-3-dependent proteolytic cleavage of protein kinase cdelta in dopaminergic cells: relevance to oxidative stress and dopaminergic degeneration. Neuroscience 119:945–964

Lakowicz JR (1999) Principles of fluorescence spectroscopy, Kluwer Academic/Plenum Publishers, New York, pp. 1–24

Li Q, Kobayashi M, Kawada T (2015) Carbamate pesticide-induced apoptosis in human T Lymphocytes. Int J Environ Res Public Health 12:3633–3645

Liu R, Siemiarczuk A, Sharom FJ (2000) Intrinsic fluorescence of the P-glycoprotein multidrug transporter: sensitivity of tryptophan residues to binding of drugs and nucleotides. Biochemistry 39:14927–14938

Lowry OH, Rosenbrough NJ, Farr AL, Randal AJ (1951) Protein measurements with the folin phenol reagent. J Biol Chem 193:265–275

Lukaszewicz-Hussain A, Moniuszko-Jakoniuk J (2004) Chlorfenvinphos, an organophosphate insecticide, affects liver mitochondria antioxidative enzymes, glutathione and hydrogen peroxide concentration. Pol J Environ Stud 13:397–401

Moller M, Denicola A (2002) Protein tryptophan accessibility studied by fluorescence quenching. Biochem Mol Biol Educ 30:175–178

Morena AJM, Serafim TL, Oliveira PJ, Madeira VMC (2007) Inhibition of mitochondrial bioenergetics by carbaryl is only evident for higher concentrations – relevance for carbaryl toxicity mechanisms. Chemosphere 66:404–411

Moreno AJM, Madeira VMC (1990) Interference of parathion with mitochondrial bioenergetics. Biochim Biophys Acta 1015:361–367

Nakagawa Y, Nakajima K, Suzuki T (2004) Chlorpropham induces mitochondrial dysfunction in rat hepatocytes. Toxicology 200:123–133

Reddy PM, Phillip GH (1992) Changes in the levels of respiration and ions in the tissues of freshwater fish, *Labeo rohita* under fenvalerate stress. Chemosphere 25:843–852

Walther DM, Rapaport D (2009) Biogenesis of mitochondrial outer membrane proteins. Biochim Biophys Acta 1793:42–51

Wu A, Liu Y (2000) Deltamethrin induces delayed apoptosis and altered expression of p53 and bax in rat brain. environ. Toxicol Pharmacol 8:183–189

Wu A, Li L, Liu Y (2003) Deltamethrin induces apoptotic cell death in cultured cerebral cortical neurons. Toxicol Appl Pharmacol 187:50–57