Effect of insecticides and inhibitors on P-glycoprotein ATPase (M-type) activity of resistant pest *Helicoverpa armigera*

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P-glycoprotein (P-gp) was detected using C219 antibodies from the membrane preparation of the insecticide resistant pest, *Helicoverpa armigera*. This protein was partially purified and found to be a glycoprotein having ATPase activity. Its molecular mass as determined by SDS–PAGE was 150 kDa. Insecticides, viz. monocrotophos, endosulfan, cypermethrin, fenvalerate and methyl parathion stimulated P-gp ATPase activity. Orthovanadate at 1.5 μM concentration inhibited P-gp ATPase activity by 70%, whereas $HgCl_2$ and PCMBS at 200 μM inhibited it by 75 and 70% respectively. EDTA and sodium azide inhibited P-gp ATPase activity by 20% at 0.5 and 2μM respectively, and sodium molybdate (10 μM) did not inhibit P-gp ATPase activity.

AMERICAN bollworm, *Helicoverpa armigera* (Hübner) is recognized as an international pest because of its cosmopolitan distribution and high damage potential, endangering more than 182 species of plants[1]. In India, annual losses to cotton and pulses alone have been estimated at US$ 300–500 million[2]. Total reliance on pesticides, including organochlorines, organophosphates, carbamates and synthetic pyrethroids to control *H. armigera* has not achieved the desired success, as it has developed resistance to several groups of chemicals[3,4]. Insects that show resistance to one insecticide generally develop resistance to another class of insecticides, a phenomenon often referred to as cross-resistance[5]. It resembles multidrug resistance (MDR), whereby resistance to one drug is accompanied by simultaneous resistance to a variety of structurally unrelated compounds.

MDR has been mainly associated with the overexpression of a 170 kDa plasma membrane protein belonging to the ATP-binding casette family. P-glycoprotein (P-gp) belongs to M-type ATPase, which renders resistance to a wide spectrum of drugs. This protein appears to cause MDR via ATP-dependent drug efflux mechanism, which prevents intracellular accumulation of drugs to an effective cytotoxic concentration in the treatment of cancer. The sequence encoding of P-gp revealed that it is a tandemly repeated molecule of about 1280 amino acids, each half consisting of a large hydrophobic domain, containing three pairs of putative membrane-spanning α-helices and a conserved hydrophilic cytoplasmic domain, containing an ATP-binding site. It has been proposed that 12 transmembrane domains associate to form a pore or channel through which P-gp actively effluxes drugs[6–8]. This P-gp-mediated MDR is thought to be an important cause of failure of cancer chemotherapy and this type of resistance occurred throughout the course of evolution[6].

Several mechanisms of resistance have been identified[3,4,9]. The presence of P-gp and its involvement in insecticide resistance has been reported in tobacco budworm, *Heliothis viresens*[5]. We have reported the presence of P-gp in *H. armigera*[9]. In the present study we report the partial purification of P-gp ATPase from the membrane preparation of insecticide-resistant pest, *H. armigera*. Further, influence of various insecticides and inhibitors on P-gp ATPase was also investigated.

Materials and methods

Chemicals

ATP, ethylene glycol, *O,O′-bis*(2-aminoethyl) *N,N,N′,N′*-tetraacetic acid (EGTA) and Ouabain were purchased from Sigma Chemical Co. (St. Louis, USA). C219 antibodies were purchased from Signet Laboratories (Dedham, MA, USA) and the ECL luminescence kit from Amersham for immunoblot. Standard marker proteins for SDS–PAGE were purchased from Bangalore Genei. Insecticides, monocrotophos (71.5%), endosulfan (94%), cypermethrin (93.2%), fenvalerate (90.5%) and methyl parathion (99.3%) were gifted by the Pesticide Analysis Laboratory, Gulbarga. All other chemicals used were of analytical grade.

Insects

Susceptible pests were obtained from Project Directorate of Biological Control, Bangalore. Resistant pests were provided by S. S. Hudikeri, Agriculture Research Station, Gulbarga. These pests have developed resistance to various insecticides[9] and were broadly referred to as the resistant population.

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Extraction of P-gp ATPase

Fifth instar larvae were washed in cold 50 mM Tris-HCl buffer (pH 7.4) and dissected to remove the gut contents. Whole body was then homogenized in 50 mM Tris-HCl (pH 7.4) containing 1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM phenyl thiourea. The homogenate was filtered through four-layered muslin cloth and centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was subjected to ultracentrifugation at 100,000 *g* for 1 h. The pellet was resuspended in the above buffer and solubilized in 1% Triton X-100 for 1 h at 4°C. Then this suspension was recentrifuged at 100,000 *g* for 30 min and the supernatant was treated with $(NH_4)_2SO_4$ to attain 40% saturation. After 2 h, the precipitate was removed by centrifugation at 15,000 *g* for 20 min. Further the supernatant was treated with $(NH_4)_2SO_4$ to attain 60% saturation and allowed to stand for 2 h. The precipitate was collected by centrifugation as above and dissolved in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM PMSF, 0.1% Triton X-100 and 1 mM EDTA. This partially purified enzyme preparation was used for further studies.

Protein concentration was determined by the method of Lowry *et al.*[10]. The presence of P-gp in partially purified preparation was confirmed using C219 antibodies[5].

ATPase activity

ATPase activity of P-gp was determined[11] by quantitating the release of inorganic phosphate from ATP. In brief, 1 ml of ATPase assay medium (aliquots of enzyme; ATP, 2.5 mM; KCl, 75 mM; $MgCl_2$, 5 mM; Tris-HCl, 50 mM; EGTA, 0.5 mM; ouabain, 2 mM; sodium azide, 3 mM; pH 7.4) was incubated for 30 min at 37°C and the reaction was terminated by addition of 2 ml ice-cold stopping medium (ammonium molybdate, 0.2% (w/v); sulphuric acid, 1.3% (w/v); SDS, 0.9% (w/v); trichloroacetic acid, 2.3% (w/v); freshly prepared ascorbic acid, 1% (w/v)) to each test tube. After 30 min incubation at room temperature, the released phosphate was quantitated colorimetrically at 660 nm. To study the effect of the above insecticides, different concentrations of insecticides were included in the reaction mixture.

Electrophorosis

SDS-PAGE was carried out on 7.5% gel, according to Laemmli[12]. Glycoprotein staining was carried by following the procedure described elsewhere[13].

Results and discussion

Initial studies were carried out to assess the activity of ATPase on the crude membranes prepared from susceptible and resistant pests. It was observed that susceptible pest membranes had low ATPase activity compared to resistant pest (Table 1). ATPase activity was slightly increased in susceptible pest compared to 50% increase in resistant pest when the membranes were solubilized in Triton X-100. This increase in activity may be induced by the detergent.

The effect of different insecticides on ATPase activity of partially purified P-gp ATPase showed that ATPase activity was stimulated by 20–35% (Table 2).

P-gp was partially purified by selective $(NH_4)_2SO_4$ precipitation. The 60% $(NH_4)_2SO_4$ fraction had most of the glycoprotein with high activity. SDS–PAGE confirmed that the presence of a high molecular mass glycoprotein (150 kDa) was over-expressed in the resistant pest, but it was not detected in the susceptible pest (Figure 1 *a*). This was further confirmed by immunoblot using C219 antibodies, which are specific for MDR1-P-gp (Figure 1 *c*). This protein when stained with Schiff's base gave a violet band, indicating that it is a glycoprotein (Figure 1 *b*).

The effect of various specific inhibitors on partially purified P-gp ATPase showed that ortho vanadate is a potent inhibitor of P-gp ATPase and inhibited ATPase activity by 70%. Other inhibitors like EDTA and sodium azide showed only 20% inhibition and sodium molybdate did not show any inhibitory effect (Table 3).

Table 2 shows that P-gp ATPase activity is stimulated by various insecticides. Crude membranes of Sf9 (insect) cells infected with baculovirus MDR1[14] exhibited high levels of vinblastin-stimulated ATPase activity ranging from 5 to 25 µmol/min mg protein. Stimulation of ATP hydrolysis by vinblastin is due to an increase in the maximal velocity

Table 1. ATPase activity in membrane preparation of *H. armigera*

Fraction	ATPase activity (nmol P_i liberated/min/mg)	
	Susceptible	Resistant
Membrane	130 ± 10.37	210 ± 11.31
Supernatant	100 ± 11.92	100 ± 10.79
Membranes solubilized in 1% Triton X-100	150 ± 14.97	280 ± 11.31

Values represent 95% confidence interval for mean activity (average ± 1.96 × SD of mean).

Table 2. Effect of different insecticides on membrane-bound and partially purified ATPase activity of resistant pest *H. armigera*

Insecticide (5 µM)	Partially purified ATPase activity of resistant strain
Control	320 ± 11.92
Monocrotophos	382 ± 13.57
Endosulfan	408 ± 10.37
Cypermethrin	401 ± 10.79
Fenvalerate	390 ± 14.97
Methyl parathion	433 ± 15.30

ATPase activity is given as nmol P_i liberated/min/mg. Values represent 95% confidence interval for mean activity (average ± 1.96 × SD of mean).

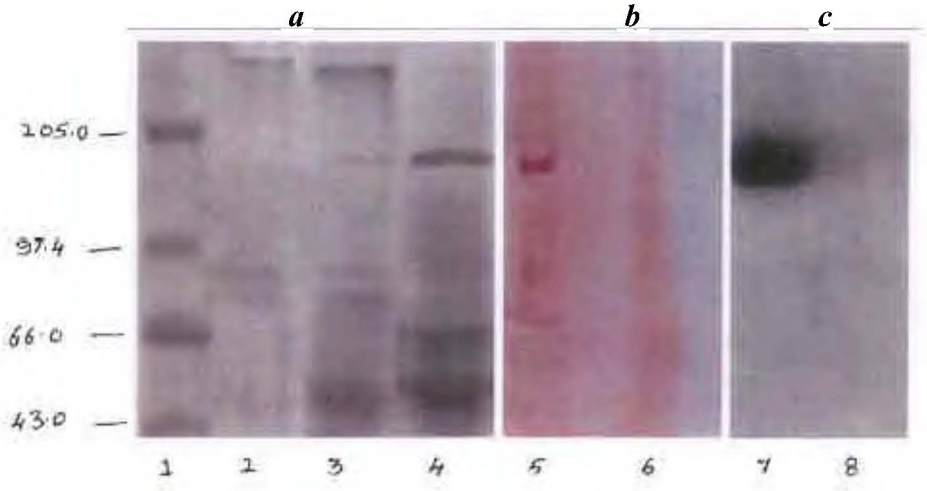

Figure 1. *a*, Coomassie blue stained SDS–PAGE. Profiles of detergent-soluble membrane proteins of *H. armigera*. Lane 1, Protein molecular weight markers, myosin, rabbit muscle (205 kDa); phosphorylase *b* (97.4 kDa); bovine serum albumin (66 kDa) and ovalbumin (43 kDa); lane 2, Whole insect extract (susceptible); lane 3, Whole insect extract (resistant); lane 4, Enriched fraction from resistant pest. In each case, 50 µg protein was loaded. ***b***, Detection of glycosylated P-gp. Lane 5, Resistant pest whole extract; lane 6, Susceptible pest whole extract. Briefly, 50 µg whole larva protein was subjected to SDS–PAGE and the foil was dried at 100°C, followed by ethanol and periodic acid treatment. The gel was washed briefly with 0.001 N HCl and placed in acidified iodine and transferred into Schiff's reagent for 20 min. During glycoprotein staining, the gel was subjected to dehydration and rehydration; hence the lanes are constricted. *c*, Detection of P-gp in resistant and susceptible pest larvae by immunoblot analysis. Lane 7, resistant pest and lane 8; Susceptible pest. In brief, 10 µg of whole larvae extract prepared from resistant and susceptible population of 4th instar larvae was resolved on 7.5% SDS–PAGE. P-gp antibodies (C219) were applied at 1 µg/ml for 2 h, followed by streptavidin horse radish peroxidase diluted to 1 : 6000 in phosphate buffered saline.

Table 3. Effect of various inhibitors on P-glycoprotein ATPase

Inhibitor	Concentration (µM)	Inhibition (%)
o-Vanadate	1.5	70
$HgCl_2$	200	75
PCMBS	200	70
EDTA	0.5	20
Sodium azide	2	20
Sodium molybdate	10	0

Experimental details are given in the text.

of ATP hydrolytic activity, without affecting the apparent K_m for ATP. Human P-gp exhibits a high-capacity drug-dependent ATP–hydrolytic activity that is a direct reflection of its drug transport capability[15]. Binding of the drug to the transmembrane region has been reported to stimulate the ATPase activity of P-gp, causing a conformational change that releases the drug to either the extracellular space or outer leaflet of the membrane, from which it diffuses into the extracellular medium[16].

Several factors responsible for insecticide resistance in *H. armigera* have been reported[9]. Presence of P-gp was detected in the resistant pest by C219 antibodies that are specific to MDR1 P-gp in *Heliothis virescens* by Lanning *et al.*[5], who demonstrated that it was involved in the insecticide resistance mechanism. The presence of P-gp (Figure 1 *c*, lane 7) in resistant pest *H. armigera* suggested its involvement in insecticide resistance.

Ortho vanadate (P-type ATPase) and SH reagents (mercuric chloride ($HgCl_2$) and p-chloromercuribenzenesulphonic acid (PCMBS)) are found to be potent inhibitors of P-gp ATPase (Table 3). Earlier, an extensive investigation on potential inhibitors of P-gp ATPase has been reported[17,18]. EDTA and ouabian are inhibitors of Ca^{2+} and Na^+K^+-ATPase respectively, but not P-gp ATPase. Also, inhibitors of F_0F_1-ATPase (mitochondrial ATPase), such as sodium azide, oligomycin and dicyclohexyldimide do not alter P-gp ATPase activity. Activity is also unaffected by inhibitors of acid phosphatases (sodium molybdate and sodium tartarate) and alkaline phosphatase (L-phenylalanine). Ortho vanadate, a classical inhibitor of many M-type ATPases, inhibits completely P-gp ATPase activity at low µM concentration (K_i = 1.5 µM)[19]. The sulphydryl reactive agents, *N*-ethylmaleimide, PCMBS and $HgCl_2$ also inhibit P-gp ATPase with K_i values in the range[19] of 80–150 µM. ATP protects P-gp ATPase from inactivation by these compounds at two sites per molecule[19,20]. A motif of all P-gp gene classes contains a conserved Cys and possibly this residue is the target for attack by sulphydryl agents.

The P-gp identified in the resistant pest is the analogue of MDR1 P-gp and is stimulated by some of the insecticides.

The function of this protein would be involved in the extrusion of insecticides, as reported in the case of *H. virescens*[5].

1. Manjunath, T. M., Bhatnagar, V. S., Pawar, C. S. and Sitanantham, S., Proceedings of the Workshop on Biological Control of Heliothis: Increasing the Effectiveness of the Natural Enemies, 11–15 November 1985, New Delhi (eds King, E. G. and Jackson, R. D.), ICRISAT, Hyderabad, 1989, pp. 197–228.
2. King, A. B. S., Heliothis/*Helicoverpa* (Lepidoptera: Noctuidae). In *Insect Pests of Cotton* (eds Matthews, G. A. and Tunstall, J. P.), CAB International UK, 1994, pp. 39–106.
3. Armes, N. J., Jadav, D. R., Bond, G. S. and King, A. B. S., Insecticide resistance in *Helicoverpa armigera* in south India. *Pestic. Sci.*, 1992, **34**, 355–364.
4. Kranthi, K. R., Jadhav, D. R., Wanjari, L. R., Alis, S. S. and Russel, D., Carbamate and organophosphate resistance in cotton pests in India 1995 to 1999. *Bull. Entomol. Res.*, 2001, **91**, 37–46.
5. Lanning, C. L., Fine, R. L., Corcoran, J. J., Ayad, H. M., Rose, R. L. and Abou-Donia, M. B., Tobacco budworm P-glycoprotein: Biochemical characterization and its involvement in pesticide resistance. *Biochim. Biophys. Acta*, 1996, **1291**, 155–162.
6. Prasad, R., Krishnamurthy, S., Prasad, R., Gupta, V. and Latha, S., Multidrug resistance: An emerging threat. *Curr. Sci.*, 1996, **71**, 205–213.
7. Gottesman, M. M. and Pastan, I., Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.*, 1993, **52**, 385–427.
8. Ralhan, R. and Kaur, J., Multidrug resistance in oral cancer. *Proc. Indian Natl. Sci. Acad., Part B*, 2003, **69**, 83–110.
9. Srinivas, R., Udikeri, S. S., Jayalakshmi, S. K. and Sreeramulu, K., Identification of factors responsible for insecticide resistance in *Helicoverpa armigera* (Hubner). *Comp. Biochem. Physiol. C*, 2004, **137**, 261–269.
10. Lowry, O. H., Rosebrough, N. J., Farr, A, L. and Randall, R. J., Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 1951, **193**, 265–275.
11. Borgnia, M., Eytan, G. D. and Assaraf, Y. G., Competition of hydrophobic peptides, cytotoxic drugs and chemosensitizers on a common P-glycoprotein pharmacophore as revealed by its ATPase activity. *J. Biol. Chem.*, 1996, **271**, 3163–3171.
12. Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophase T_4. *Nature*, 1970, **227**, 680–685.
13. Gerard, C., *Methods in Enzymology* (eds Murnay, P. and Dautsche, P.), Academic Press, New York, 1990, vol. 182, pp. 529–539.
14. German, U. A., Willingham, M. C., Pastan, I. and Gottesman, M. M., Expression of human multidrug transporter in insect cells by a recombinant *Baculovirus*. *Biochemistry*, 1990, **29**, 2295–2303.
15. Scarborough, G. A., Drug-stimulated ATPase activity of the human P-glycoprotein. *J. Bioenerg. Biomembr.*, 1995, **27**, 37–41.
16. Ramachandra, M., Ambudkar, S. V., Chen, D., Hrycyna, C. A., Dey, S., Gottesman, M. M. and Patan, I., Human P-glycoprotein exhibits reduced affinity for substrates during a catalytic transition state. *Biochemistry*, 1998, **37**, 5010–5019.
17. Doige, C. A., Yu, X. and Sharom, F. J., ATPase activity of partially purified P-glycoprotein from multidrug-resistant *Chinese hamster* ovary cells. *Biochim. Biophys. Acta*, 1992, **1109**, 149–160.
18. Senior, A. E., Al-Shawi, K. M. and Urbartsch, I. L., The catalytic cycle of P-glycoprotein. *FEBS Lett.*, 1995, **377**, 285–289.
19. Al-Shawi, M. K. and Senior, A. E., Characterization of the adenosine triphosphatase activity of Chinese hamster P-glycoprotein. *J. Biol. Chem.*, 1993, **268**, 4197–4206.
20. Urbartsch, I. L., Al-Shawi, M. K. and Senior, A. E., Characterization of the ATPase activity of purified Chinese hamster P-glycoprotein. *Biochemistry*, 1994, **33**, 7069–7076.

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