ATPase Natural Inhibitor, Purification And Its Interaction With Pesticide Effect.

M.Massoud, M.Abdei-Ghany and Nader Shaker.
Pesticide Chemistry Dept. College of Agriculture, University of Alexandria, Alex. Egypt.

Abstract.

The natural ATPase inhibitor was extracted from both honey bee thorax and beef heart by exposing the purified ATPase enzyme to heat treatment followed by centrifugation and purification of the supernatant by using DEAE-Sephadex chromatography.

The molecular weight of honey bee ATPase natural inhibitor was calculated as 8000 dalton and appears in one protein band using sodium dodecylsulfate-slab polyacrylamide gel electrophoresis(SDS-PAGE).

The inhibitory effect of the extracted crude and purified natural ATPase inhibitors were studied against their F_1 or mitochondrial ATPase. The interaction of natural inhibitor with ATPase activity was compared kinetically with gossypol (natural botenical material) and dicofol (pesticide). Natural ATPase inhibitor showed uncompetitive interaction with ATP substrate which differed from the recorded noncompetitive effect of gossypol and dicofol on ATPase activity. The interaction between natural ATPase inhibitor in presence of gossypol or dicofol on ATPase activity was studied.

Introduction.

ATPase is the primary energy source for most biological processes. All biological systems have elaborated what appear to be natural peptide regulators of H^+-ATPases. Its function was studied in beef heart (Horstman and Racker,1970), in yeast (Satre et al,1975), in bacteria (Lastras and Munoz,1974)and in chloroplast (Nelson et al,1972).

Mitochondria inhibitor protein plays a regulatory role in oxidative phosphorylation. It controls the backflow of energy from ATP to energy - linked processes but has a little effect on ATP synthesis. Natural inhibitor is an essential component of the system catalyzing oxidative phosphorylation.

This study is an approach to isolate ATPase natural inhibitor from insect source and to study its characteristic role to interact with other natural materials (gossypol) or pesticide (dicofol) on mitochondrial-soluble ATPase activity.

Materials & Methods.

Honey bee Apis mellifera workers were obtained from the experimental station of the Faculty of Agriculture, University of Alexandria. Bovine heart was obtained from Alexandria slaughter house within 1-2 hrs after butchered the animal.

Preparation of $F_1$and mitochondrial-ATPase; Two hundred thoraxes of honey bee workers were collected and placed in an ice coold 30 mM tris-HCl buffer pH 7.4 containing 0.25M sucrose. The tissue was homogenized and filtered through a double layer of cheese cloth. The filterate was centrifuged at 800xg for 10 min at 4 °C. The pellets were suspended in tris-sucrose buffer. The suspension was quick frozen and thawed several times and then stored at -20 °C as stock of

mitochondrial ATPase activity. Also the bovine heart mitochondrial ATPase was prepared(5).

F_1-ATPase was extracted from insect mitochodrial fraction(6) and from bovine heart(7). Protein content for the extracted tissues was determined by the bio-rad method(8).

Extracti n of ATPase natural inhibitor by incubating 20 ml of insect or bovine heart mitochondria protein in a boiling water bath for 10 min. Then centrifuged at 12000xg for 5 min. The pellets were discarded and the sup rnatant was used as a crude natural inhibitor.

This endogenous inhibitor protein was purified by using a small column of DEAE-Sephadex ion exchange chromatography(4) with slightly modified technique(9).

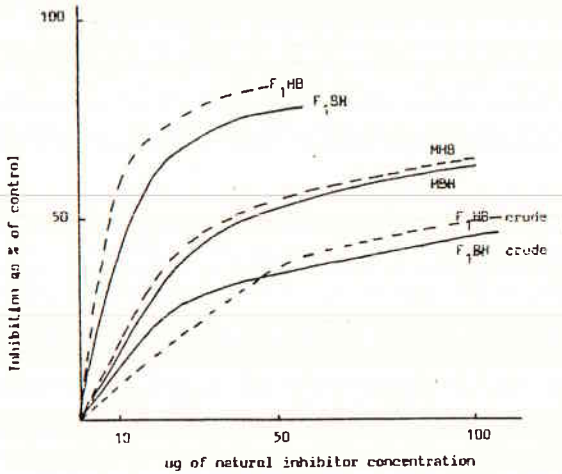
Identification of natural inhibitor molecular weight was determined by using SDS-PAGE & coomassie blue staining method(10).

Assessment of F_1- and mitochondarial - ATPase activity and inhibition was carried out using the optimum condition(11).

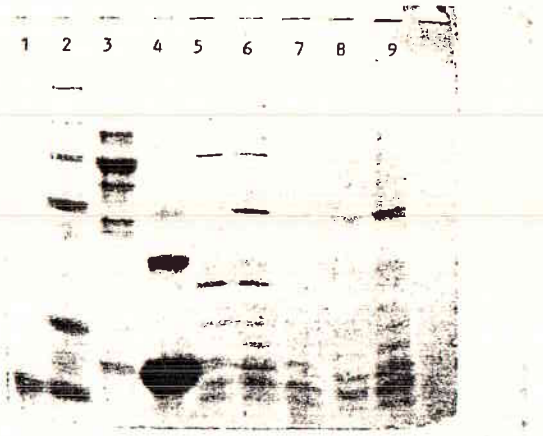
Results and Discussions.

ATPase natural inhibitor extracted from honey bee or beef heart was heat stable, nondialyzable and soluble in water. The activity of the natural ATPase inhibitor is related to a type of active peptide protein. Its activity is completely destroyed by brief exposure to trypsin (Table,1) & ref.9,12,13.

The effect of extracted crude and purified natural inhibitor for honey bee and beef heart were tested against their sources of mitochondrial or soluble ATPase(Fig,1). The inhibitory effect of the pure form of the natural inhibitor was more potent to affect its source of soluble ATPase activity.This agree with which was found before by Dianoux et al(14). Also the experimental study shows that the natural



Fig(1); Effect of natural ATPase inhibitor protein on honey bee (--HB) and beef heart(—BH) soluble (F_1) and mitochondrial (M) ATPase activity.



Fig(2): photographic copy of electrophoretic pattern of natural inhibitor protein extracted from honey bee and beef heart by using SDS-PAG method.

inhibitor which was isolated from insect mitochondria had no effect on beef heart mitochondrial ATPase activity and vice versa.

Table(1): Trypsin effect on the natural crude inhibitor activity.

Reaction tube content	Natural crude inhibitor			
	Honey bee		Beef heart	
	S.A	%I	S.A	%I
Mitochondrial ATPase activity.....	1.6	0.0	1.9	0.0
ATPase + Trypsin + Trypsin inhibitor.....	1.58	1.3	1.8	5.2
ATPase + Natural inhibitor + Trypsin + Trypsin inhibitor.....	1.6	0.0	1.9	0.0
ATPase + Trypsin + Trypsin inhibitor + Natural inhibitor.....	0.9	44.0	0.9	50.0
ATPase + Natural inhibitor.....	0.93	42.0	0.9	50.0

Trypsin used in experiment is Sigma brand type 1 extracted from bovine pancreas contain 10000 units BAEE per mg protein.

Identification of the extracted natural inhibitor was carried out by passing the crude extract through DEAE-Sephadex ion exchange column chromatography. Using the eluted purified protein for molecular weight determination by SDS-PAGE method. The honey bee natural inhibitor appeared as one clear band No,1 with 8000 dalton molecular weight estimated by using the mobility of the standard molecular weight proteins. Also the photograph of the gel electrophoresis shows the mobility of the crude natural ATPase inhibitor before passing through the ion exchange purification column No,2. Also honey bee F_1-ATPase was illustrated in column No3. Honey bee natural ATPase inhibitor was compared with beef heart crude natural inhibitor column No 5,6,8 & 9 and after ion exchange chromatography purification column No 7.

The beef heart mitochondrial ATPase protein has been isolated and characterized with regard to physical and chemical properties to five subunits (15). The 5th subunit was identified as ATPase inhibitor peptide. This peptide is basic protein removed from the ATPase molecule in the form of monomer(5700) and dimer(1 350) molecular weights. The conversion of the dimer to monomer or trimer was dependent on the presence of agents capable of reducing disulfide bonds. This agrees with data illustrated in Fig(2) which shows that the purified honey bee natural inhibitor gave one band.

Comparative study was made between the purified natural ATPase inhibitor of honey bee and beef heart against F_1-ATPase. Honey bee purified natural inhibitor was seven times more potent than the crude form to inhibit honey bee F_1-ATPase. Also for the beef heart purified natural inhibitor was three times more potent to inhibit F_1-ATPase than the crude form(Fig 1). This is may be due to,that inhibitor protein not require coupled processes to do its activity but it can do its effect in a loosely coupled submitochondrial particles(16).

Gossypol affected honey bee and beef heart F_1-ATPase in a range from (0.0 - 4.0 uM = 0.0 - 2.0 ug) Fig(3) which is 10 times lower than the effective concentrations of the natural inhibitor. This data can clear the natural inhibitor which affect ATPase activity under controlled condition can couses other effect on the cell under uncontrolled condition looks like gossypol which affect ATPase activity and couses other effects on the cell activity(17&18).

Dicofol inhibitory effect was in between both natural compounds. It reached its maximum effect at

7.4 ug(4 uM), then started to decrease Fig(4).This agree with which was found before by Balba(19).

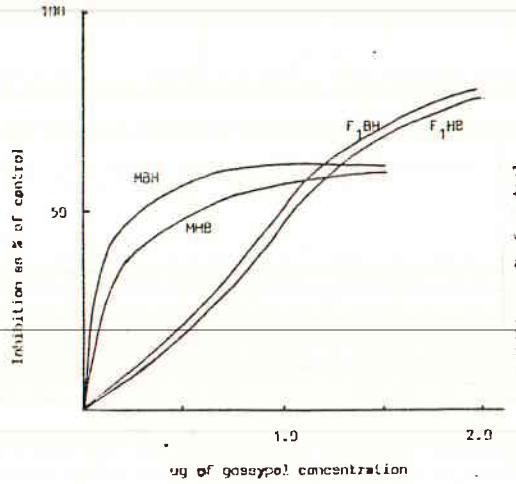
Kinetics of either the natural inhibitor,or gossypol or dicofol interaction with honey bee and beef heart F_1-ATPase activity was studied. Natural inhibitor decreased the value of Vmax and Km giving a parallel lines with F_1-ATPase activity in honey bee and beef heart. The type of inhibition was classified as uncompetitive inhibition in which the inhibitor was able to bind to the enzyme substrate complex but not to the free enzyme. The substrate,enzyme, inhibitor combination can only dissociate to yeild the inhibitor(I) and enzyme substrate complex with an equilibrium constant Ki (Table 2).

The kinetics of gossypol interaction with honey bee and beef heart F_1-ATPase and dicofol interaction with honey bee and beef heart mitochondrial-ATPase were studied. It was clear that gossypol & dicofol decreases the value of the maximum F_1 or mitochondrial ATPase activity without altering the Km value (Table 2). This type of inhibition is known as noncompatitive inhibition in which inhibitor has no effect on substrate binding and vice versa(19 & 20).

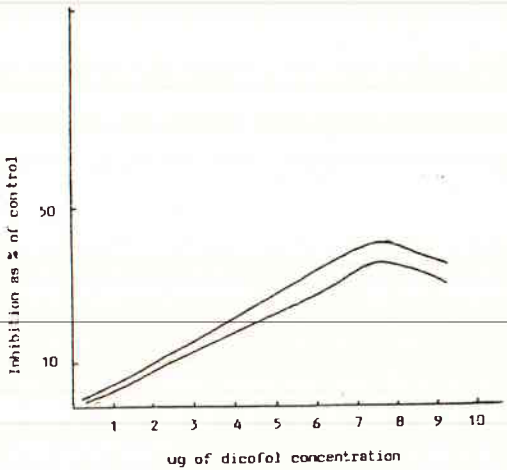
Table(2); Kinetic study for natural inhibitor, gossypol and dicofol interaction with honey bee and beef heart soluble and mitochondrial ATPase activity.

ATPase activity	Vmax uM Pi/mg Protein/min		Km mM		Ki uM/mg prot./min	type of inhibition.
Honey bee	8.02	5.00*	.66	.40*	0.00	
+natural inhibitor	4.34		.357		0.82	uncompatitive
+gossypol	4.34		.66		1.50	noncompatitive
+dicofol		0.285*		.40*	2.5	noncompatitive
Beef heart	2.86	2.63*	.31	.66*	0.00	
+natural inhibitor	1.66		.20		1.20	uncompatitive
+gossypol	4.76		.66		1.50	noncompatitive
+dicofol		2.08*		.66*	1.50	noncompatitive

* Kinetic of dicofol studied on mitochondrial ATPase.



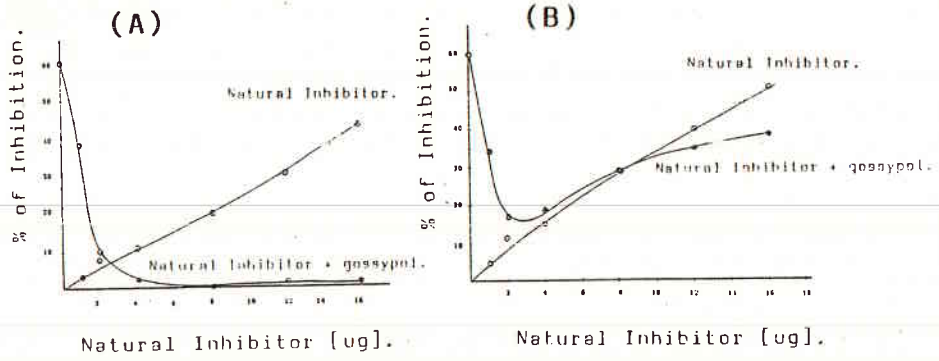
Fig(3); Effect of gossypol on honey bee (HB) and beef heart (BH) soluble (F_1) and mitochondrial (M) ATPase activity.



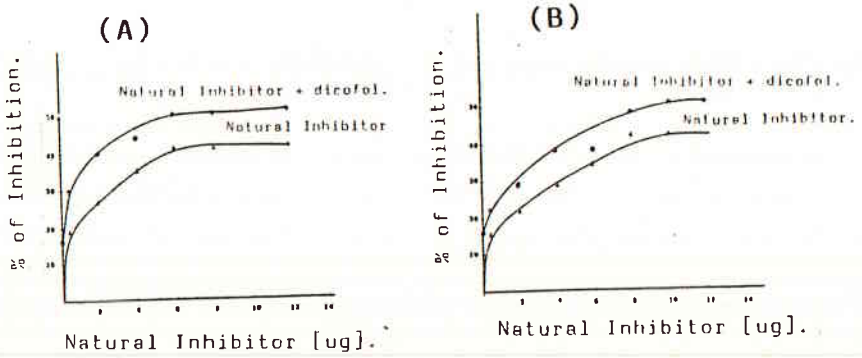
Fig(4); Effect of dicofol on honey bee (HB) and beef heart (BH) soluble (F_1) and mitochondrial (M) ATPase activity.

Interaction of gossypol with natural inhibitor affected by honey bee and beef heart F_1-ATPase ativity showed in Fig(5) that gossypol-natural inhibitor combination interact each other effect on honey bee F_1-ATPase with antagonistic manner..While with beef heart F_1-ATPase, gossypol antagonise the inhibitory effect of natural inhibitor at lower concentration however,gossypol effect is negligable at higher concentration of the natural inhibitor.This effect may be due to the different changes coused by natural inhibitor on proteins which changes its interaction with gossypol(21).

Interaction of dicofol with natural inhibitor affect honey bee and beef heart mitochondrial-ATPase activity. Fig(6). Dicofol effect seems to be additive effect. This result shows that both inhibitors can affect different sites of action(22). This indicates that during the steady state of phosphorylation only limited numbertof ATPase molecules



Fig(5); Inhibition of honey bee(A) and beef heart(B) F_1-ATPase activity in presence of 2uM gossypol at different concentration of purified natural inhibitors.



Fig(6); Inhibition of honey bee(A) and beef heart(B) mitochondrial ATPase activity in the presence of 2uM dicofol at different concentrations of purified natural inhibitors.

are in the active catalytic state,and that only during active hydrolysis of the inhibitor protein interacts with its inhibitory site, rather than interacting with an intermediate state of the enzyme that appears either during the synthetic or hydrolytic reactions. This can clear the interaction of gossypol and dicofol with natural inhibitor affect ATPases active sites.

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الملخص العربى

تم استخلاص و تنقيه البروتين المثبط الطبيعى لانزيم الادينوزين ثلاثى الفوسفاتيز مصدر الطاقه فى النظم الحيويه كذالك التعرف على وزنه الجزيئى وذلك باستخدام التحليل الكروماتوجرافى بالهجره الكهربائيه حيث ظهر فى صوره بروتين منفرد ذات وزن جزيئى ٨٠٠٠ دالتون و ذلك بالنسبه للبروتين المستخلص من صدور نحل العسل و تمت مقارنته بمثيلـه المعزول من قلب البقر.

اظهرت النتائج قدره هذا المثبط الطبيعى المستخلص من صدور نحل العسل او قلب البقر على تثبيط نشاط كل من انزيم الادينوزين ثلاثى الفوسفاتيز الذائب عشره امثال قدرته على تثبيط الانزيم المرتبط.

عند استعمال تركيزات منخفضه من الجوسيبول وجد انها تضاد اثـر المستخلص البروتينى على تثبيط انزيم الادينوزين ثلاثى الفوسفـاتيز الذائب المستخلص من كل من نحل العسل او قلب البقر بينما عند زياده تركيز المثبط البروتينى الطبيعى تبدأ فى ظهـور اثرها مره اخرى.

يتداخل مبيد الدايكوفول مع المثبط البروتينى الطبيعى على تثبيط انزيم الادينوزين ثلاثى الفوسفاتيز المرتبط بقدره ثابته تبعا للتركيز المستخدم من المبـيد.

درست ميكانيكيه التثبيط لتفاعل البروتين المثبط الطبيعى على مصدره من انزيم الادينوزين ثلاثى الفوسفاتيز الذائب حيث اظهرت اثرها مـن النوع التنافسى بينما يتداخل الدايكوفول مع الانزيم المرتبط بطـريقـه لاتنافسيه لماده التفاعل وكذلك الجوسيبول يتداخل بطريقه لاتنافسيـه مع ماده التفاعل على الانزيم الذائب.