Int. J. Environ. Res. Public Health **2008**, *5(2)*, 120-124

International Journal of
Environmental Research and Public Health
ISSN 1661-7827
www.ijerph.org

Juvenile Hormone Analogues, Methoprene and Fenoxycarb Dose-Dependently Enhance Certain Enzyme Activities in the Silkworm Bombyx Mori (L)

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Received: 26 October 2007 / Accepted: 30 April 2008 / Published: 30 June 2008

Abstract: Use of Juvenile Hormone Analogues (JHA) in sericulture practices has been shown to boost good cocoon yield; their effect has been determined to be dose-dependent. We studied the impact of low doses of JHA compounds such as methoprene and fenoxycarb on selected key enzymatic activities of the silkworm *Bombyx mori*. Methoprene and fenoxycarb at doses of 1.0 µg and 3.0fg/larvae/48 hours showed enhancement of the 5th instar *B. mori* larval muscle and silkgland protease, aspartate aminotransaminase (AAT) and alanine aminotransaminase (ALAT), adenosine triphosphate synthase (ATPase) and cytochrome-c-oxidase (CCO) activity levels, indicating an upsurge in the overall oxidative metabolism of the *B.mori* larval tissues.

Keywords: Juvenile hormone analogues, methoprene, fenoxycarb, silkworm bombyx mori (l), larva.

Introduction

Juvenile hormone analogues (JHA) are known to prolong the larval life in insects, and these have been long utilized for the improvement of silk production in the silkworm *Bombyx mori (L).* As early as 1971, Akai et al. [1], demonstrated enhanced accumulation of silk protein accompanying the prolongation of larval growth in *Bombyx mori* treated with JHA [2]. In the last two to three decades, a number of newer JHA compounds have been investigated; and many investigators have tried to study the effect JHA compounds have on various hybrids of silkworms to elucidate the contribution of varied JHA formulations in increasing the yield. Many of these experiments also attempt to explain the mode of action of individual formulations [3-7]. In view of the biological significance of JHA compounds' on the yield of silk material this study was undertaken in an attempt to study the effect of selected JHA compounds, methoprene and fenoxycarb on certain key enzyme activity profiles in tissues of the silkworm *B. mori.*

Materials and Methods

A hybrid race PM x NB_4D_2 of the silkworm *Bombyx mori* was used in the present investigation. The rearing conditions as given by Sonwalker [8] were followed to nuture the silkworm larvae.

Chemicals

The JHA analogues, methoprene (isopropyl(2E,4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadinoate) and fenoxycarb (6-ethyl-N-[2-(4-phenoxy-phenoxy) ethyl] carbamate] both of 98.5% purity were selected for the study.

Treatment of Animals

Previous report from our laboratiory indicate that a dose of 1.0 µg/larvae of methoprene and 3.0fg/larvae of fenoxycarb induced an increase in yield of the *Bombyx mori* larval growth period by 15 and 10 hours respectively

[5]. Same doses in acetone were topically applied to the 5th instar *B. mori* larvae on day one and two. Following treatment, on day 4th and 6th muscle and silkgland tissues were isolated and used for enzymatic assays. The experimental control groups received equal amounts of acetone and untreated group of larvae were considered as normal control.

Assay of Enzymatic Activities

In the control and experimental *B. mori* larval muscle and silkgland the protease activity was measured by the method of Moore and Stein [9], total ATPase activity by the method of Fritz and Hamrick [10] and as modified by Desaiah and Ho [11]. Aspartate aminotransaminase (AAT) and Alanine Aminotransaminase (ALAT) activities were assayed by adopting the procedures as reported by Reitman and Frankel [12] and the cytochrome-c-oxidase (CCO) activity by the procedure as given by Oda et al. [13].

Statistical Analysis

The mean of individual observations (for both control and experimental groups) were taken into consideration. Statistical significance of the data was analyzed through two way ANOVA (Analysis of variance); SNK (Student Newman-Keuls) test and regression analysis [14].

Results

Protease activity in methoprene treated *B. mori* 5th instar larval muscle and silkgland showed an increasing trend over the corresponding experimental group of tissues and the increment was found to be statistically significant ($p<0.001$) over the experimental control (Table 1). Similar trends were also observed for fenoxycarb treated *B. mori* larval tissues, however the day 6 silkgland showed enhanced increase in its protease activity (from 53.41 in the control to 65.70 µM tyrosine/mg protein/hr in experimental group; Table 2).

The data related to the effect of methoprene and fenoxycarb on *B. mori* muscle and silk gland AAT and ALAT activities were presented in tables 1 and 2, where both the agents at the doses employed significantly increased both the enzymatic activities in the muscle and silk gland over their corresponding experimental control groups of tissues. Increased total ATPase and cytochrome-c-oxidase activities were observed in the muscle and silkgland tissues treated with methoprene and fenoxycarb (Table 3 and 4) and the changes were found to be statistically significant over the experimental control group of tissues ($p<0.001$). For any enzymatic activity assayed, more percent increase was observed in methoprene treated B. mori larval muscle and silkgland compared to those of fenoxycarb treated ones (Table 1-4).

Table 1: Methoprene induced changes of Protease, AAT and ALAT levels in the Silkworm *Bombyx mori.*

Name of the enzyme	*4th day*						*6th day*					
	Muscle			*Silk gland*			*Muscle*			*Silk gland*		
	Control	*Expt. Control*	*Test*	*Control*	*Expt. Control*	*Test*	*Control*	*Expt. Control*	*Test*	*Control*	*Expt. Control*	*Test*
Protease (µM tyrosine/mg protein/hr)	58.85	59.02	68.44	42.50	43.65	55.40	70.95	72.00	85.22	50.50	51.66	69.07
SD	±0.842	±1.05	±0.91	±1.42	±0.80	±2.16	±1.05	±0.83	±2.91	±0.75	±2.33	±1.27
PC		(0.29)	(15.96)		(2.71)	(26.92)		(14.80)	(18.36)		(2.30)	(33.70)
T		NS	P<0.001		NS	P<0.001		NS	P<0.001		NS	P<0.001
AAT (µM pyruvate/mg protein / hr)	0.54	0.57	0.81	0.36	0.38	0.53	0.69	0.68	0.95	0.54	0.56	1.14
SD	±0.04	±0.03	±0.04	±0.22	±0.08	±0.05	±0.11	±0.08	±0.11	±0.06	±0.08	±0.08
PC		(4.96)[a]	(41.15)[b]		(4.93)[a]	(37.60)[b]		(-2.46)[a]	(40.44)[b]		(3.703)[a]	(103.57)[b]
T		NS	P<0.001		NS	P<0.001		NS	P<0.001		NS	P<0.001
ALAT (µM pyruvate/ mg protein/hr)	2.16	2.29	3.95	1.74	1.74	2.99	3.95	3.99	5.48	3.25	3.27	4.16
SD	±0.31	±0.10	±0.06	±0.02	±0.02[a]	±0.03	±0.030	±0.04	±0.04	±0.07	±0.05	±0.06
PC		(6.02)[a]	(72.49)[b]		(0)	(71.83)[b]		(1.01)[a]	(37.34)[b]		(0.61)[a]	(27.22)[b]
T		NS	P<0.001		NS	P<0.001		NS	P<0.001		NS	P<0.001

Each value is the mean ± SD of 20 samples, PC: Percent change, NS: Not Significant, Expt.: Experimental

a: Percent change over normal control

b: Percent change over experimental control

Table 2: Fenoxycarb induced changes of protease, AAT and ALAT levels in the silkworm *Bombyxmori. L.*

Name of the enzyme	*4th day*						*6th day*					
	Muscle			*Silk gland*			*Muscle*			*Silk gland*		
	Control	*Expt. Control*	*Test*	*Control*	*Expt. Control*	*Test*	*Control*	*Expt. Control*	*Test*	*Control*	*Expt. Control*	*Test*
Protease (µM tyrosine/mg protein/hr)	61.95	62.85	70.04	43.66	44.08	44.61	63.00	64.00	66.00	52.26	53.41	65.70
SD	±2.33	±1.20	±0.86	±1.24	±2.08	±0.98	±1.90	±2.20	±2.19	±2.11	±0.86	±1.22
PC		(1.45)[a]	(11.44)[b]		(0.96)[a]	(1.20)[b]		(1.59)[a]	(3.12)[b]		(2.20)[a]	(23.01)[b]
T		NS	P<0.001		NS	P<0.005		NS	P<0.001		<0.005	P<0.001
AAT (µM of pyruvate/mg protein/hr)	0.552	0.570	0.762	0.372	0.369	0.461	0.83	0.84	0.92	0.561	0.57	1.03
SD	±0.056	±0.02	±0.049	±0.036	±0.04	±0.05	±0.12	±0.04	±0.07	±0.15	±0.08	±0.02
PC		(3.26)[a]	(33.68)[b]		(-0.80)[a]	(19.95)[b]		(1.70)[a]	(10.01)[b]		(1.24)[a]	(80.34)[b]
T		NS	P<0.001		NS	P<0.001		NS	P<0.001		NS	P<0.001
ALAT (µM pyruvate/mg protein/hr)	2.39	2.41	4.52	1.78	1.76	2.94	4.61	4.55	5.01	3.96	4.02	5.00
SD	±0.04	±0.02	±0.02	±0.036	±0.04	±0.07	±0.13	±0.10	±0.073	±0.04	±0.15	±0.10
PC		(0.83)[a]	(87.55)[b]		(-1.12)[a]	(67.05)[b]		(-1.30)[a]	(10.11)[b]		(1.51)[a]	(24.37)[b]
T		NS	P<0.001		NS	P<0.001		NS	P<0.001		NS	P<0.001

Each value is the mean ± SD of 20 samples, PC: Percent change, NS: Not Significant, Expt.: Experimental
a: Percent change over normal control
b: Percent change over experimental control

Table 3: Effect of methoprene on total ATPase and Cytochrome-C-Oxidase activity level of *Bombyx mori.* L.

Name of the enzyme	*4th day*						*6th day*					
	Muscle			*Silk gland*			*Muscle*			*Silk gland*		
	Control	*Expt. Control*	*Test*	*Control*	*Expt. Control*	*Test*	*Control*	*Expt. Control*	*Test*	*Control*	*Expt. Control*	*Test*
Total ATAase (µM pi/mg protein/hr)	14.19	15.23	19.69	16.04	16.13	17.52	17.01	18.26	24.90	21.50	22.14	29.07
SD	±1.00	±1.59	±1.90	±0.93	±0.68	±1.25	±0.89	±0.53	±0.84	±0.90	±1.33	±0.92
PC		(7.33)[a]	(29.28)[b]		(0.56)[a]	(8.62)[b]		(7.35)[a]	(36.36)[b]		(2.98)[a]	(31.30)[b]
T		NS	P<0.001		NS	P<0.001		NS	P<0.01		NS	P<0.001
Cytoctrome C-oxidase (µg formazan/mg protein/hr)	20.85	21.41	23.59	24.21	24.36	27.04	23.15	24.03	25.21	36.60	35.78	40.62
SD	±0.80	±0.62	±1.95	±1.07	±0.84	±0.83	±1.66	±0.85	±1.07	±2.35	±1.66	±0.83
PC		(2.66)[a]	(10.18)[b]		(0.62)[a]	(11.00)[b]		(3.80)[a]	(4.91)[b]		(-2.24)[a]	(13.52)[b]
T		NS	P<0.001		NS	P<0.001		NS	P<0.001		NS	P<0.001

Each value is the mean ± SD of 20 samples, PC: Percent change, NS: Not Significant, Expt.: Experimental
a: Percent change over normal control
b: Percent change over experimental control

Table 4: Effect of Fenoxycarb on total ATPase and Cytochrome-c-oxidase activity levels in the silkworm Bombyx *mori.* L.

Name of the Enzyme	*4th day*						*6th day*					
	Muscle			*Silk gland*			*Muscle*			*Silk gland*		
	Control	*Expt. Control*	*Test*	*Control*	*Expt. Control*	*Test*	*Control*	*Expt. Control*	*Test*	*Control*	*Expt. Control*	*Test*
Total ATPase (µM pi/mg protein/min)	12.82 ±0.86	12.64 ±0.71	16.36 ±1.22	8.07 ±0.64	7.96 ±0.43	12.41 ±0.61	14.74 ±0.91	16.21 ±1.24	20.77 ±1.03	13.60 ±0.82	14.06 ±0.50	18.92 ±1.75
SD		(-1.40)[a]	(29.43)[b]		(-1.36)[a]	(55.90)[b]		(9.97)[a]	(28.13)[b]		(3.38)[a]	(34.56)[b]
PC		NS	P<0.001		NS	P<0.001		NS	P< 0.001		NS	P<0.001
T												
Cytochrome-C Oxdase (µg of formazan/mg protein/hr)	21.16 ±0.72	22.50 ±1.07	23.05 ±0.88	24.90 ±0.90	24.11 ±0.56	26.71 ±0.75	24.05 ±0.91	24.48 ±1.70	29.06 ±0.61[b]	36.82 ±0.93	36.25 ±2.10	38.33 ±1.06
SD		(6.33)[a]	(2.44)[b]		(-3.17)[a]	(10.78)[b]		(1.78)[a]	(18.71)		(-1.54)[a]	(5.73)[b]
PC		NS	P<0.001		NS	P< 0.001					NS	P<0.001
T												

Each value is the mean ± SD of 20 samples, PC: Percent change, NS: Not Significant, Expt.: Experimental
a: Percent change over normal control
b: Percent change over experimental control

Discussion

In the literature, much was emphasized in relation to the impact of JHA's on economic profiles of various breeds of silkworms; practically there is little or no literature as to how various JHA's alter the key enzyme activities of *B.mori* during its growth period. To cover this, the authors tried to provide experimental basis on the fate of selected enzymatic activities involved in protein and energy metabolism in the muscle and the silk gland tissue of *B. mori.* Silk production basically depends on the *B. mori* larval protein metabolism which in turn needs more energy generating events, spinning requires more muscular activity and silk is being produced by the silkgland. On these lines, the selection of enzymes involved in protein and energy metabolism as well as tissues like muscle and silk gland in the present study is justifiable.

This increases in the levels of methoprene and fenoxycarb treated *B. mori* larval tissue protease AAT, ALAT, ATPase and cytochrome-c-oxidase activity levels indicating an elevation in the oxidative metabolism of proteins and energy metabolism (Table 1-4).

Protease basically catalyzes the breakdown of proteins resulting in the formation of free amino acids (FAA). Present study clearly demonstrated that JHA treatment of 5th instar *B. mori* larval muscle and silk gland show higher protease activity. Further, with the increase in growth there is an increase in the protease activity of *B. mori* larval tissues (Table 1 and 2). Similar findings have previously been reported for the hormone thyroxine [15]. Transamination has been found to be one of the mechanisms, whereby the balance between amino acid pool and protein synthesis were regulated [16]. Our results for the control muscle and silk gland confirms data presented by Pant & Kumar who demonstrated that that ALAT exhibits higher activity than AAT through embryonic development of the silkworm [17]. At the doses and time intervals employed both methoprene and fenoxycarb increased levels of the *B. mori* 5th instar muscle and silk gland AAT and ALAT (transaminases) suggesting elevated transamination activities. Both of these enzymes act as link enzymes and contribute to an increase in TCA cycle.

The elevated levels of AAT and ALAT in *B. mori* larval tissue indicate that the JHAs favor the use of the gluconeogenesis pathway in energy production. Similar results and conclusions have been reported for various JHA compounds [5, 18-21].

Phosphatases and ATPases are known to be complex enzyme systems [20]. Protein synthesis and storage mechanisms are energy consuming phenomena and the supply of ATP is considered to be an important factor for these processes [22]. The role of cytochrome-c-oxidase (CCO) in energy generation, and its relation to oxidative metabolism versus hormone regulatory events in insect species have been extensively reviewed by several authors [23-25]. We found in the current studies that methoprene and fenoxycarb enhanced total ATPase activity of the *B. mori* larval muscle and silk gland tissue, a finding which confirms reports from other authors. The explanation given is that there may be an increase in enzyme mass or increase in membrane related transport function, others believe that it and may be for the purpose of energy generation that contribute to the synthesis and secretion of silkprotein [23-25]. It is well established that animals tissues possess the pathway of electron transfer capable of conserving ATP

through oxidative metabolism [26]. Increase in *B. mori* larval tissue cytochrome-c-oxidase under JHA stress reflects an increase in the oxidative metabolism ultimately leading to the generation of more ATP to meet energy demands of individual tissues. Thus, it may be envisaged that methoprene and fenoxycarb, by way of enhancing *B. mori* larval tissue ATPase and cytochrome-c-oxidase activity, may contribute to generation of more ATP necessary for the larva for its synthetic and developmental processes.

The results from our studies shows that JHA compounds methoprene and fenoxycarb at the doses of 1.0 μg and 3.0fg/larvae/48 hours respectively stimulate proteolysis, transamination and energy metabolism of the *Bombyx mori* larval tissues and these in turn may contribute to energy generation events in the JHA treated *B. mori* larvae. Methoprene induced the highest percent increases compared to that of fenoxycarb. Our findings contribute, in part to the understanding of the mode of action of methoprene and fenoxycarb on the silkworm *Bombyx mori*.

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