J. Agric. Sci. Mansoura Univ., 33 (3): 2253 - 2260, 2008

DETECTION OF FREE AMINO ACIDS AND ISOENCYMES FROM THE HAEMOLYMPH OF THE MATURE LARVAL IMSTAR OF SILKWORM, *BOMBYX.MORI L.*

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ABSTRACT

Seventeen different free amino acids were detected from haemolymph of the mature larval instar of *Bombyx mori* L. male and female. Three amino acids (proline, glycine and glutamine) represent 77.1 and 69.18 % of all pool amino acids found in females and males haemolymph, respectively. Proline was the most predominated free amino acids in males (45.81 µ mole/ ml) and females (55.25 µ mole/ ml). Glycine came second with a concentration of 27.58 and 30.25 µ mole/ ml in males and females, respectively. The multiple forms of the isozyme glucose-6-phosphate dehydrogenase (G-6-PDH) in mature larval instar haemolymph of both sexes of *B. mori* are pentameric, while that of the isoenzyme alkaline phosphatase (ALP) are trimeric. Three isoenzyme bands of isoenzyme G-6-PDH are characteristic of males (RF. 0.10, 0.043 and 0.068) and three other bands (RF. 0.05, 0.49 and 0.88) are characteristic of females. For the isoenzyme ALP has a band with RF. 0.051 characterizing of males while the band which had RF. 0.58 characterizes females.

INTRODUCTION

The silkworm, *Bombyx mori* L., has a high economic importance in sericulture. Fibroins are the protein substance excreted by various species of Arthropoda from special glands and stored there in solution perior to extrusion to form filaments. These filaments were embedded in a globular protein; sericin. The fibroin of *B. mori* is unusual in its amino acid composition and it was given by Sasaki and Noda (1973). The fibroins that provide the structural basis of the cocoons of different insects species perform essentially the same function for each (protection from predators, extremes of temperature, physical shock, microbiological attack and the general rigors of climate). It is remarkable that the variation in amino acid composition that has been found is perfectly compatible with this function (Agosin, 1978).

Metabolism in insects is limited by three ways; the glycolysis, the tricarboxylic acids cycle and the pentose cycle. These cycles are controlled by dehydrogenase enzymes which play many important roles in insect body and their relative activities may be related to the function and energy-yielding demands of the tissue (Horiey, 1967). Studies have shown that differential isozyme expression is the result of different synthetic rates (Davis and McIntyre, 1998). The isozyme alkaline phosphatase (ALP) plays an important role in desorption of metabolites generally and sugars in particular (Sridhara and Bhat, 1963 and Rousell, 1971). This may indicate its important role in

transportation of materials like glucose through the intestinal wall. This work is presented to through more light on amino acid concentration and detection of isoenzymes; glucose-6-phosphate dehydrogenase (G-6-PDH) and alkaline phosphatase (ALP) in last larval instar of *B. mori* haemolymph.

MATERIALS AND METHODS

1- Determination of free amino acids:

The haemolymph of *B.mori* larvae were collected from punctured pro-abdominal legs of thirty healthy mature larvae of both sexes in sterile tubes with a small crystal of phenylthiourea to prevent melanization of the sample. Three ml of haemolymph sample was added to 10 ml ethanol at room temperature, then the precipitated protein was filtered and evaporated to dryness at 40 ºC. The residue was dissolved in 3 ml water. 0.5 ml dansylchloride solution was added to 1 ml of the conditioned sample and adprested to pH 8.0 with the prepared sodium bicarbonate solution. For the derivatization-reaction the solution is placed in the dark at room temperature for 16 hours. The reaction solution was transferred with acetone : water (7 : 3) in 10 ml measuring flask and filled up. For the calibration standard, 1mg/ml of standard mixture dissolved in water. 1 ml of dansylchloride solution was added to 1 ml amino acid and filled up to 100 ml with acetone : water (7 : 3) after derivatization reaction. Concentration of the standard solution in 10 µg / µl.

Sample application: S1 U1, S2 U1, S3 U1, S4 U2, S5 U2, S6 U2.
S1 = 1 µl = 10 ng absolute, S2 = 2 µl = 20 ng absolute, S3 = 3 µl = 30 ng absolute, S4 = 4 µl = 40 ng absolute, S5 = 5 µl = 50 ng absolute, U1 = 6 µl (Sample diluted 1 : 10), U2 = 5 µl, S = Standard, U = Sample.

Developing the plates with solvents according to the method adopted by Das and Sawant (1993). Two types of solvents were incorporated in the separation of amino acids and they determine the retention factor values (hRf value). Solvent 1 is chloroform : propinic acid : ethyl acetate (23 : 6 : 4) for the separation of Dansyl-L-arginine, Dansyl-L-lycine, Dansyl-L-cystine, Dansyl-L-aspartic acid, Dansyl-L-serine, Dansyl-L-threonine, Dansyl-L-glutamic acid, Dansyl-L-glutamine. Solvent 2 is chloroform : acetone : propinic acid (24 : 10 : 5) for the separation of Dansyl-L-tyrosine, Dansyl-L-alanine, Dansyl-L-methionine, Dansyl-L-valine, Dansyl-L-proline, Dansyl-L-phenylalanine, Dansyl-L-isoleucine, Dansyl-L-leucine.

CAMAG HPTLC sanner II with Lab Data system and CATS evaluation software was used for densitometic evaluation.

2. Isoenzymes assay:

The enzymes, glucose-6-phosphate dehydrogenase (G-6-PDH) and alkaline phosphatase (ALP) in blood supernatants were separated by discontinuous polyacrylamide gel electrophoresis according to Maurer (1968) and were assayed and detected by the methods of Shaw and Parsad (1970). Enzymatic protein bands were designated according to the system

nomenclature proposed by Shaklee *et al*. (1990). Electrophoresis was carried out conveniently in discontinuous polyacrylamide gels (stacking and tracking gels). An amount of 50 µl of clear supernatant of the blood for each sample was mixed with 20 µl of protein dye (1% bromophenol blue) and 20 µl of 2% sucrose. 30 µl of the mixture per gel slot was used to be applied per each sample for isoenzymes electrophoresis. After electrophoresis, the gel was transferred into a staining solution (50 – 70 ml) which was then replaced by a destaining mixture of methanol, acetic acid and water (5: 1: 5 v/v/v). A potential gradient of 20 v/cm across the gel was applied for 4 hours at 8 ºC.

RESULTS AND DISCUSSION

Haemolymph free amino acids

Seventeen different free amino acids were detected from haemolymph of the last larval instar of *Bombyx mori* L.. male and female (Table 1). These amino acids were arranged in 4 groups; I, II, III, IV on the basis of polar versus non-polar character *i. e*., polar and charged, polar and uncharged, non polar and those with unique properties (Karp, 1969).

Group I contained aspartic, glutamine, lysine, arginine and histidine. The concentration of these acids were 40.264 and 28.154 µ mole/ ml in the last larval instar of male's and female's haemolymph, respectively. The amino acid glutamine was the highest (23.404 µ mole/ ml in males and 17.080 µ mole/ ml in females). The most predominant amino acids of this group are glutamine followed by histidine, aspartic acid, arginine and lysine in both sexes. The members of this polar and changed amino acids represent 28.95% of the total amino acids present in male's haemolymph and 21.14% in female's haemolymph.

Group II contained serine, threonine, and tyrosine. The concentration of these acids was 11.950 and 7.752 µ mole/ ml in the last larval instar of male's and female's haemolymph, respectively. Their percentages were 8.33 and 5.07% of total amino acids in male's and female's haemolymph, respectively. The most abundant amino acids in this group were threonine followed by serine and tyrosine in both sexes.

Group III contained six free amino acids *i. e*., phenylalanine, methionine, leucine, isoleucine, alanine and valine. They constitute 8.33 and 6.99 % of the total pool of the last larval instar amino acids haemolymph of males and females, respectively. The concentrations of the individual amino acids ranged between 2.655 and 0.821 µ mole/ ml in males while it ranged between 2.345 and 0.741 µ mole/ ml in females.

Group IV includes proline, glycine and cystine. They have unique properties that separate them from the other amino acids. The R group of glycine consist of only a hydrogen atom. Proline is unique in having its α-amino group as part of a ring (making it an amino acid). Cystine contains a reactive sulphydral (-SH) group and is often present covalently linked to another cystine residue as a disulphide (-SS-) bridge. Disulphide bridges often form between two cystines that are distant from one another in the polypeptides backbone or even in two separate polypeptides. This group

constituted 54.37 and 66.03 % of the total pool of the last larval instar amino acids haemolymph of males and females, respectively.

When these amino acids were arranged according to their concentration in haemolymph, four groups were obtained as indicated in Table (2). The first group contains amino acids with concentrations higher than 10 µ mole/ ml (3 amino acids for both sexes) while the second group includes concentrations lower than 10 to 5 µ mole/ ml (2 amino acids in both sexes). The third group has concentrations lower than 5 to 1 µ mole/ ml (10 amino acids in males and 9 in females) but the last group includes concentrations are less than 1 µ mole/ ml (2 amino acids in males and 3 in females).

Table (1). Free amino acids concentrations (µ mole/ ml) in haemolymph of *B. mori* mature larval instar.

Groups of amino acids	Amino acids	Free amino acids (µ mole/ ml)	
		Males	Females
Group I Polar and charged	(A) Glutamine	23.404	17.080
	(B) Histidine	7.676	4.505
	(A) Aspartic acid	3.964	2.326
	(B) Arginine	3.254	2.256
	(B) Lysine (E)	1.966	1.987
	Total percentage	40.264	28.154
Group II Polar and uncharged	Threonine (E)	4.050	2.377
	Serine	6.551	4.499
	Tyrosine	0.989	0.876
	Total percentage	11.590	7.752
Group III Non polar	(N) Alanine	1.245	1.103
	(N) Valine (E)	0.821	0.741
	(N) leucine (E)	2.541	2.222
	(N) Isoleucine (E)	1.810	0.813
	(N) Methionine (E)	2.513	2.082
	(N) Phenylalanine (E)	2.655	2.345
	Total percentage	11585	9.306
Group 1 R-group with unique properties	(N) Proline	27.578	30.251
	Glycine (E)	2.220	2.446
	(N) Cystine	45.814	55.254
	Total percentage	75.612	87.951
	Total amino acid pool	139.051	133.163

A = Acidic, N = Neutral, B = Basic, E = Essential amino acid

Table (2). Total free amino acids (µ mole/ ml) in haemolymph of *B. mori* mature larval instar according to their concentration.

Amino acid groups	Males		Females	
	Amino acid	Concentration	Amino acid	Concentration
Group I Concentration > 10 µ mole/ ml	Proline	45.814	Proline	55.254
	Glycine	27.578	Glycine	30.251
	Glutamine	23.404	Glutamine	17.080
	Total	96.796	Total	102.585
Group II Concentration < 10 to 4.1 µ mole/ ml	Histidine	7.676	Histidine	4.505
	Threonine	6.551	Threonine	4.499
	Total	14.227	Total	9.004
Group III Concentration < 4.1 to 1 µ mole/ ml	Aspartic acid	3.964	Aspartic acid	2.326
	Serine	4.050	Serine	2.377
	Aginine	3.254	Aginine	2.256
	Alanine	1.254	Alanine	1.103
	Methionine	2.513	Methionine	2.084
	Cyctine	2.220	Cyctine	2.446
	Isoleucine	1.012	leucine	2.222
	leucine	2.541	Phenylalanine	2.345
	Phenylalanine	2.655	Lysine	1.987
	Lysine	1.966	------	------
	Total	25.420	Total	19.104
Group IV Concentration < 1 µ mole/ ml	Tyrosine	0.989	Tyrosine	0.876
	Valin	0.821	Valin	0.741
	------	-----	Isoleucine	0.813
	Total	1.810	Total	2.430
Total pool	138.253		133.123	

Isoenzymes:

The isoenzymes were detected by electrophoresis. The number of recorded bands for glucose-6-phosphate dehydrogenase (G-6-PDH) in last larval instar haemolymph of both sexes of *B. mori* are listed in Table (3) and the electrophoretic patternswere shown in figure (1).

This isoenzyme showed 5 bands in both males and females but only bands which have R.F. 0.31 and 0.68 were common inboth sexes. Bands with R.F 0.10, 0.43 and 0.96 were found to be characteristic for males while bands with R.F 0.053, 0.49 and 0.88 are characteristic for females.The present results show that females last larval instar have higher enzymatic activity of G-6-PDH than in males, which may suggest that the pentose cycle accelerates the metabolic process of glucose in females more than in males. The multiple forms of isozyme G-6-PDH in the haemolymph of the last larval instar of *B. mori* are pentameric in both sexes. This enzyme appears to be the predominated key enzyme for the proceeding of the pentose metabolism in insects, and it is mainly active in the fat body (Horiey, 1967).

Three protein bands have been observed for the isozyme alkaline phosphatase (ALP) in males and females (Table, 4 and figure, 2). The band which have R.F 0.51 is characteristic to males while band which have R.F

0.58 is representative for females. Bands with R.F 0.27 and 0.83 are are common and shared in males and females.

The results show that females last larval instar have higher enzymatic activity of ALP than in males The multiple forms of isozyme ALP in the haemolymph of the last larval instar of *B. mori* are trimeric in both sexes.

Table (3). Activities of glucose-6-phosphate dehydrogenase (G-6-PDH) in haemolymph of 5th instar larvae of both sexes of *B. mori*.

Bands	Males		Females	
	R.F	% amount	R.F	% amount
1	-	-	0.053	23.1
2	0.10	18.8	-	-
3	0.31	18.3	0.31	22.2
4	0.43	17.8	-	-
5	-	-	0.49	16.8
6	0.68	22.3	0.68	19
7	-	-	0.88	18.8
8	0.94	22.6	-	-
Sum		99.9		99.9
In lane	100		100	

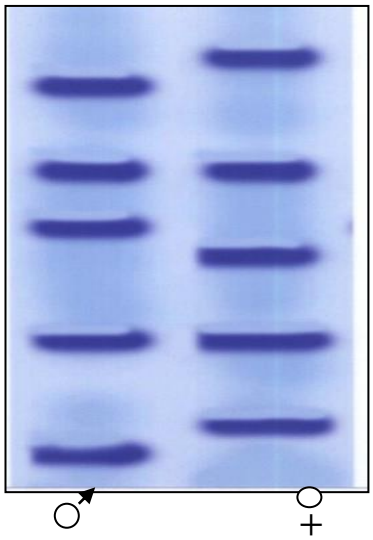

Fig. 1. Electrophoretic pattern of glucose-6-phosphate dehydrogenase isozyme in (G-6-PDH) in haemolymph of 5th instar larvae of both sexes of *B. mori*.

Table (4). Activities of alkaline phosphatase (ALP) in haemolymph of 5th instar larvae of both sexes of *B. mori*.

Bands	Males		Females	
	R.F	% amount	R.F	% amount
1	0.27	30.9	0.27	27.7
2	0.51	31.1	-	-
3	-	-	0.58	19.2
4	0.83	38	0.83	53
Sum		100		99.9
In lane	100		100	

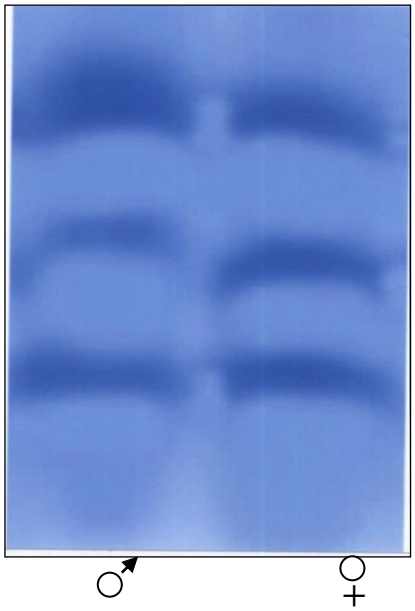

Fig.2. Electrophoretic pattern of alkaline phosphatase isozyme (ALP) in haemolymph of 5th instar larvae of both sexes of *B. mori*.

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الكشف عن الأحماض الأمينية الحرة والمشابهات الإنزيمية فى دم اليرقات تامة النمو لدودة الحرير التوتية *Bombyx mori* L.

محمد أحمد عيد، سعد بن عايض العتيبي ، صلاح عبد الله صالح المعصراوي و محمد رفعت أبو العلا

قسم الحشرات الاقتصادية والمبيدات – كلية الزراعة – جامعة القاهرة – الجيزة – مصر .

تم الكشف عن ١٧ نوع من الأحماض الأمينية الحرة فى دم البرقات تامة النمو لذكور وإناث دودة الحرير التوتية . ثلاثة أنواع من الأحماض الأمينية (برولين ، جليسين ، جلوتامين) وجدت بنسبة٧٧,١ % من مجموع الأحماض الأمينية فى الإناث يقابلها ٦٩,١٨ % فى الذكور . الحامض الأميني برولين كان هو السائد حيث وجد بنسبة ٤٥,٨١ ميكرومول / ميلي لتر فى حالة الذكور وبنسبة ٥٥,٢٥ ميكرومول / ميلي لتر فى حالة الإناث . الحامض الأميني جليسين كان فى المرتبة الثانية بتركيز ٢٧,٥٨ و ٣٠,٢٥ ميكرومول / ميلي لتر فى كلا من دم برقات الذكور والإناث على الترتيب. المشابه الإنزيمي G-6 – PDH وجد فى دم يرقات كلا الجنسين فى الصورة الخماسية بينما وجد المشابه الإنزيمي ALP فى صورة ثلاثية . وجدت ثلاث أشرطة فى تحليل المشابه الإنزيمي G-6 – PDH فى الذكور (RF 0.10, 0.043, 0.068) بينما كانت فى الإناث (RF o.o5, 0.49 , 0.88).المشابه الإنزيمي ALP وجد فى صورة شريط (RF o.o51) فى الذكور و (RF 0.58) فى الإناث .

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