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α- and β-Amylase Isozyme Expresser Native Proteins in Tropical Silkworm *Bombyx mori* L.

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Amylase isozyme based three multivoltine *viz.*, N+*p*, N*p*, N+p^{cho} and two bivoltine-D6+*p*, D6*p* syngenic lines (Syn. L) were developed from germplasm (GP) stocks Nistari (N) and D6 respectively. Haemolymph isozyme pattern at pH 7.0 and 8.5 depicted a total 11 number ($Amy^{1\ to\ 6}$ at pH 7.0 and $Amy^{1\ to\ 5}$ at pH 8.5) of native proteins (NP) of various sizes are amylase isozyme expressers. Among eleven NPs, two NPs of 770 kDa (Amy^6 at pH 7.0) and 376 kDa (Amy^3 at pH 8.5) are α-amylase expressers and remaining NPs of 370, 364, 350, 329 and 274 kDa at pH 7.0 and 206, 292, 416, 725 kDa at pH 8.5 are β-amylase expressers. Accordingly, digestive juice amylase isozyme pattern at aforesaid pH also depicted a total number of 10 NPs ($Amy^{1\ to\ 5}$) at each pH 7.0 and 8.5 are amylase expressers of which NP of 387 kDa (Amy^4 at pH 7.0) and 780 kDa (Amy^5 at pH 8.5) are α-amylase expresser. Remaining NPs of 338, 297 & 216 kDa at pH 7.0 and 370, 341, 329 & 302 kDa at pH 8.5 are β-amylase expresser. Recurrent backcross lines (RBL) *viz.*, N+*p*RBL and N*p*RBL were developed through introgression of high shell weight character (a multigenic trait) to be used further for congenic line (Con. L) development and to understand any association with introgressed character. Isozyme pattern in haemolymph of RBLs depicted only one α-amylase of 770 kDa at pH 7.0 and 376 kDa at pH 8.0 with three and four respective β-amylase bands but in bivoltine lines numbers of β-amylase bands vary between 1 to 2 at aforesaid pH. Variability was also observed in digestive juice of multivoltine and its RBLs but bivoltine lines express null activity at both pH except appearance of one very week α-amylase band D6+p at pH 8.5. Overall study suggests that not a single NP at both pH is common for expression of any band of amylase isozyme *i.e.*, a totally different set of proteins are the amylase isozyme expresser at specific pH and no molecular factor of amylase is associated in developed RBLs which showed improvement on survival, single cocoon shell weight (SCSW) and single filament length over receptor parents.

Key words: Native protein (NP), α- and β-amylase, Syngenic line (Syn. L), Introgression, Recurrent backcross line (RBL)

Introduction

Among the polysaccharides, amylase is one of the most important enzymes for carbohydrate metabolism in plants and animals stated by Ainsworth *et al.* (1987) and Gale and Ainsworth (1984). In silkworm, some previous results (Matsumura, 1934a, b; Ito, 1960; Hirata, 1971, 1974; Tanaka and Kusano, 1980; Gamo, 1983; Hara *et al.*, 1984, 1986) also considered amylase as one of the biochemical parameters. Hirata (1974) and Kuroda (1979) studied the association between specific biochemical parameters like digestive juice amylase and blood α-ketoglutaric acid and yield parameters in silkworm *B. mori*. Wajirokera *et al.* (1984) studied the activity and polymorphism of digestive juice in various strains of *B. mori*. Day and Waterhouse (1953) reported the existence of β-amylase in haemolymph and mid gut tissue in silkworm. In tropical silkworm, a little information on amylase were provided by Chatterjee *et al.* (1988, 1992, 1993) and Chatterjee and Dutta (1992). They had done multiple regression analysis and stated that amylase activity in digestive juice had a

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positive correlation with survivability (ERR) and a negative correlation with larval weight, larval duration, cocoon weight and shell weight. Thereafter, not much attention was paid to study the existence and association of β-amylase in haemolymph and digestive juice of tropical silkworm *B. mori.* In the present study our interest is to study whether β-amylase exists in haemolymph or digestive juice and has any association with survival or yield contributing parameters of tropical silkworm, *B. mori*, which are under discussed.

Materials and Methods

Insect

The silkworm breeds Nistari (N) and D6 were obtained from the germplasm (GP) bank and rearing was conducted at different seasons by following standard rearing method. Continuous selection was done phenotypically (larval marking, cocoon shape, cocoon colour and hibernating character of the eggs) and physiologically (scoto- phase duration in different stages of development -2^{nd}, 3^{rd} and 4^{th} and maturation period for spinning). Three multivoltine lines and two bivoltine lines were separated.

Collection of digestive juice

Twenty to twenty five larvae on 5^{th} day of 5^{th} instar were taken from each line and kept for one-hour starvation. Each larva was exposed to chloroform vapour for a brief time before the collection of digestive juice individually. The moment the larva started regurgitating, digestive juice was collected (approximately 100 – 200 µl) in a chilled microfuge tube coated with 1% EDTA to preserve the enzymatic stability. The collected samples were centrifuged at 2511 × g for 10 min at 4°C. The supernatant was placed in EDTA coated tubes and stored at 20°C.

Collection of haemolymph

Prolegs of the same larvae were punctured with a sterilized needle. The haemolymph samples (approximately 200 µl from multivoltine breed and 300 µl from bivoltine breed) were collected separately from each larva of each line, immediately after the collection of digestive juice, in chilled microfuge tubes coated with 0.1% phenylthiouria to prevent the melanization. The injured prolegs were sealed immediately with liquid paraffin to stop further bleeding and to keep the larvae alive for further screening and rearing. The supernatant was collected in 0.1% PTU coated microfuge tubes after centrifugation at 2511 × g and stored at –20°C.

Polyacrylamide gel electrophoresis (PAGE)

Eight µl haemolymph and 10 µl digestive juice of each un-boiled and boiled sample were electrophoresed separately under non-denatured conditions on a 7.7% polyacrylamide gel prepared. Boiling of sample was done at 70°C for 15 min only to inactivate the β-amylase activity (Nighikara and Masahiro, 1971). PAGE was run with haemolymph and digestive juice samples separately along with native protein (NP) molecular markers in a separate lane to determine the isozyme expresses (only anodic) native proteins (NPs) at pH 7.0 and 8.5 with a constant current of 3 mA per lane and till the tracing dye reach up to the target distance. After electrophoresis 5 mM $CaCl_2$, 50 mM Tris-HCl mediation solution (pH 6.5) was added with 0.75% hydrolyzed starch. This radical substance solution in the gel was soaked and incubated with constant agitation on a rotary shaker at 37°C for 45 min. After washing in distilled water, the solution of 0.1N potassium iodide (KI) was added to the gel and stained for 1 – 2 min and then washed in distilled water. Based on the reaction between potassium (KI) solution and starch, gel exhibited deep brown background with colour less amylase band described by Banno *et al.* (1984). Lane used for native protein markers was cut and stained separately following the standard methodology. Gels were documented in the System GDS-7600-UVP Ltd. U.K. and recorded photographically. Relative mobility (R_f) was calculated for each polypeptide from the formula: R_f = Distance of protein migration divided by distance of dye migration (Shi and Jackowski, 1998) and each R_f value of protein marker bands were plotted on spread graph sheet to make a standard curve. The R_f value of each isozyme band is plotted against the standard curve to determine the molecular weight of enzyme expresser NP.

Syngenic line (Syn. L) development

Larvae possessing homogeneous (phenotipical, physiological and biochemical- isozyme banding for amylase enzyme) were selected together in each line from successive generation and allowed to sib mating for nine generations to develop syngenic lines. After fixation of desired characters, three multivoltine *viz.*, N+*p*, N*p*, N+p^{cho} and two bivoltine - D6+*p*, D6*p* were developed as syngenic lines (Chattopadhyay *et al.*, 2001a, b, c).

Recurrent backcross line (RBL) development

Two crosses were made between developed Syn.Ls N+*p* (receptor) × D6+*p* (donor) and N*p* (receptor) × D6*p* (donor) parent to introgress the genes for high shell weight. Cocoons were selected those having high shell weight and other phenotipical characters like larval marking, cocoon shape, colour and voltinism, etc., as receptor parent from

the cross (F_1). Thereafter, consecutive back crosses were conducted for five generations in a similar fashion and finally sib mating was performed between male and female moths emerged from pupae with maximum homogeneity like receptor parent and high shell weight, closer to donor parent resulting in RBL (Chattopadhyay *et al.*, 2001a, b). But after backcrossing for five generations and finally sib mating depicted that the transgression of target character was taken place over receptors but not completely dragged in RBLs, these lines were considered here

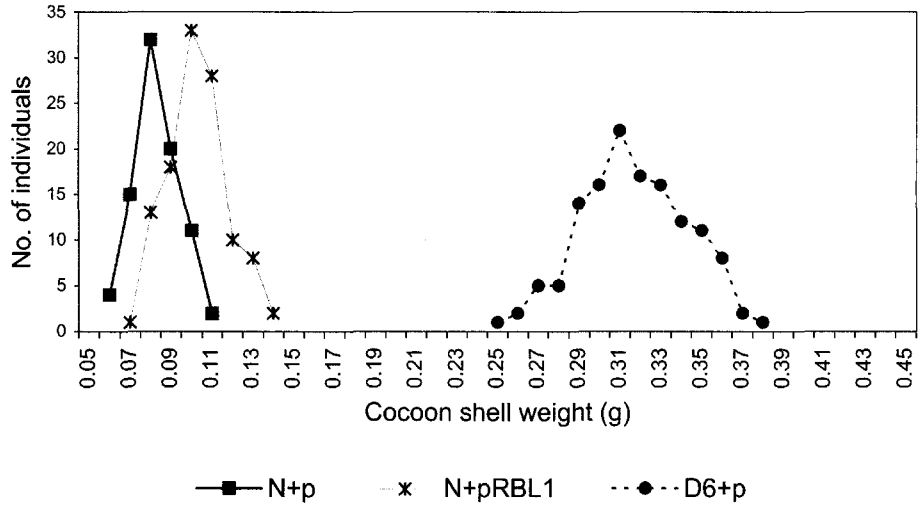

Fig. 1. Transgression of single cocoon shell weight in RBL1 over receptor N+*p* through frequency distribution, where D6+*p* is a donor.

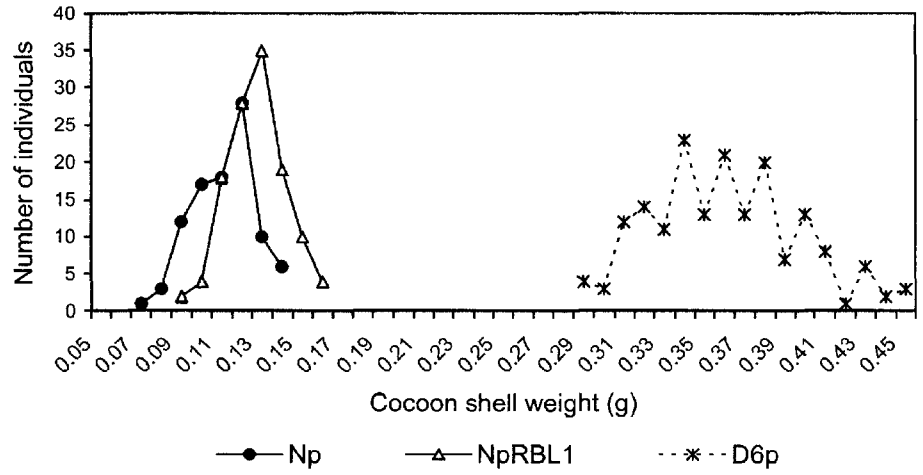

Fig. 2. Transgression of single cocoon shell weight in RBL1 over receptor N*p* through frequency distribution, where D6*p* is a donor.

Table 1. Rearing performance of developed syngenic and recurrent backcross lines and their parents during unfavourable season

Line	Cocoon yield/ 10,000 larvae (no.)	Cocoon yield/10,000 larvae (kg.)	Pupation Rate (%)	SCW (g)	SCSW (g)	Cocoon shell percent (%)	Filament length (mts)	Denier (pts)
N*p*RBL	7133	5.925	72.33	0.856	0.117	13.77	425	1.69
N*p*	6440	5.204	64.40	0.799	0.110	13.94	310	1.57
N+*p*RBL	5800	4.858	58.80	0.816	0.102	12.67	395	1.75
N+*p*	6520	4.903	66.40	0.717	0.082	11.61	305	1.63
N+p^{Cho}	6419	4.328	64.52	0.661	0.081	12.22	355	1.63
N (GP)	5027	3.582	50.27	0.663	0.079	11.82	285	1.59
D6 (GP)	2218	2.569	22.18	1.687	0.290	17.19	772	2.66

as N+*p*RBL and N*p*RBL (Fig. 1 and 2, Table 1). The quantitative characters were considered to find out any association of amylase isozyme pattern with survival, weight (g) of a single cocoon (SCW), weight (g) of a single cocoon shell (SCSW), cocoon shell percentage (SR%).

Results

Anodic amylase isozyme pattern of haemolymph at pH 7.0 and 8.5 depicted six and five amylase isozyme bands with variable number of bands in different lines at respective pH. The NP of 770 kDa is only one and common protein band to all as α-amylase where as, native protein 274, 329, 350, 364 and 370 kDa are β-amylase expresser at pH 7.0 (Table 2, Fig. 3). Accordingly 376 kDa native protein is only one and common protein band to all as α-amylase expresser and other native proteins 208, 292, 416, 725 kDa are the β-amylase expresser at pH 8.5, though only the polymorphic behaviour was recorded in Nistari (GP) line where 416 and 725 kDa native proteins are α-amylase expresser (Table 2, Fig. 4).

Anodic digestive juice amylase isozyme pattern exhibited five each isozyme bands which were variable in numbers, specially in multivoltine lines but interestingly null in bivoltine lines at both pH 7.0 and 8.5. The native protein 216, 297, 338 kDa are β-amylase expressers where as, 387 and 582 kDa are α-amylase expressers at pH 7.0 (Table 3, Fig. 5). On the other hand native proteins 302, 329, 341 and 370 kDa are the β-amylase expressers and

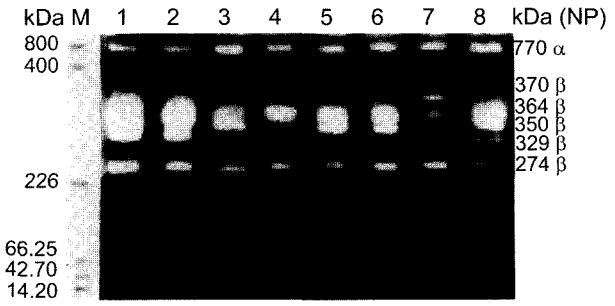

Fig. 3. Vertical 7.7 PAGE of anodic α- and β-amylase isozyme pattern in haemolymph at pH 7.0 and the molecular weight (kDa) of native protein as an expresser of α- and β-amylase isozyme. M: Marker, Lane 1, N(GP); Lane 2, N+*p*; Lane 3, N+*p*RBL1; Lane 4, D6+*p*; Lane 5, N*p*; Lane 6, N*p*RBL1; Lane 7, D6*p*; Lane 8, N+p^{Cho}.

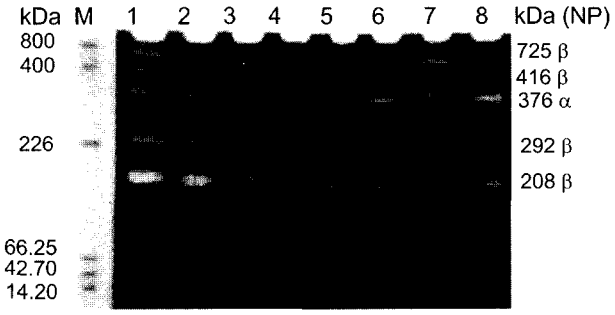

Fig. 4. Vertical 7.7 PAGE of anodic α- and β-amylase isozyme pattern in haemolymph at pH 8.5 and the molecular weight (kDa) of native protein as an expresser of α- and β-amylase isozyme. M: Marker, Lane 1, N(GP); Lane 2, N+*p*; Lane 3, N+*p*RBL1; Lane 4, D6+*p*; Lane 5, N*p*; Lane 6, N*p*RBL1; Lane 7, D6*p*; Lane 8, N+p^{Cho}.

Table 2. Molecular weight (kDa) of native protein as α- and β-amylase isozyme expressers in haemolymph at pH 7.0 and 8.5 in different lines of Nistari and D6

Band	kDa	N(GP)	N+*p*	N+*p*RBL1	D6+*p*	N*p*	N*p*RBL1	D6*p*	N+p^{Cho}
pH 7.0									
Amy^6	770	α	α	α	α	α	α	α	α
Amy^5	370	–	–	–	–	–	–	β	–
Amy^4	364	β	β	β	β	β	β	β	β
Amy^3	350	β	β	β	–	β	β	–	β
Amy^2	329	β	β	–	–	–	–	–	β
Amy^1	274	β	β	β	β	β	β	β	β
pH 8.5									
Amy^6	-	–	–	–	–	–	–	–	–
Amy^5	725	α	β	β	β	β	β	β	β
Amy^4	416	α	–	β	–	β	β	–	–
Amy^3	376	α	α	α	α	α	α	α	α
Amy^2	292	β	β	β	–	β	β	β	β
Amy^1	208	β	β	β	–	β	β	–	β

Table 3. Molecular weight (kDa) of native protein as α- and β-amylase isozyme expressers in digestive juice at pH 7.0 and 8.5 in different lines of Nistari and D6

Band	KDa	N(GP)	N+*p*	N+*p*RBL1	D6+*p*	N*p*	N*p*RBL1	D6*p*	N+p^{Cho}
pH 7.0									
Amy^5	582	α	–	–	–	–	–	–	–
Amy^4	387	α	α	α	–	–	α	–	α
Amy^3	338	β	–	β	–	–	–	–	–
Amy^2	297	β	–	β	–	–	β	–	–
Amy^1	216	β	–	–	–	–	–	–	β
pH 8.5									
Amy^5	780	α	–	α	α	α	α	–	α
Amy^4	370	–	–	β	–	–	–	–	–
Amy^3	341	β	β	β	–	β	–	–	β
Amy^2	329	–	–	β	–	–	β	–	–
Amy^1	302	β	–	β	–	β	β	–	β

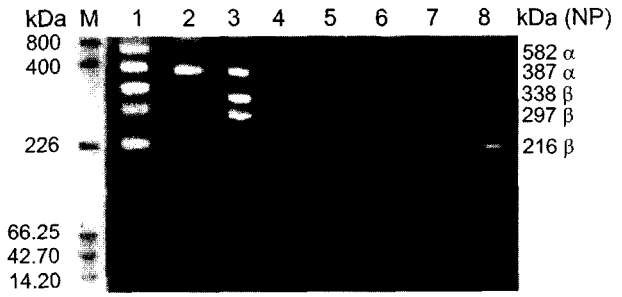

Fig. 5. Vertical 7.7 PAGE of anodic α- and β-amylase isozyme pattern in digestive juice at pH 7.0 and the molecular weight (kDa) of native protein as an expresser of α- and β-amylase isozyme. M: Marker, Lane 1, N(GP); Lane 2, N+*p*; Lane 3, N+*p*RBL1; Lane 4, D6+*p*; Lane 5, N*p*; Lane 6, N*p*RBL1; Lane 7, D6*p*; Lane 8, N+p^{Cho}.

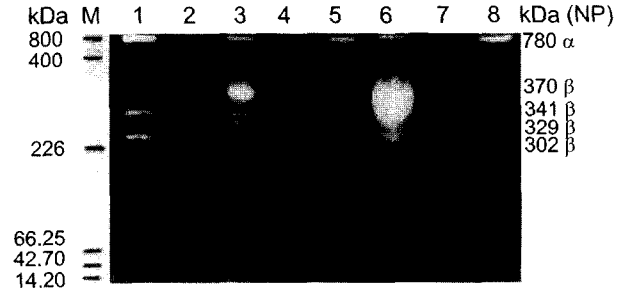

Fig. 6. Vertical 7.7 PAGE of anodic α- and β-amylase isozyme pattern in digestive juice at pH 8.5 and the molecular weight (kDa) of native protein as an expresser of α- and β-amylase isozyme. M: Marker, Lane 1, N(GP); Lane 2, N+*p*; Lane 3, N+*p*RBL1; Lane 4, D6+*p*; Lane 5, N*p*; Lane 6, N*p*RBL1; Lane 7, D6*p*; Lane 8, N+p^{Cho}.

780 kDa is only the α-amylase expresser at pH 8.5 (Table 3, Fig. 6). Beside the target character SCSW of N*p* and N+*p* transgressed from 0.110 g to 0.123 g and 0.082 g to 0.115 g respectively. Similarly the filament length of RBLs also increased from 310 mts to 425 mts and 305 mts to 359 mts respectively (Table 1).

Discussion

Amylase isozyme pattern in haemolymph and digestive juice though highlighted the polymorphism in number of α and β-amylase bands at different pH yet in haemolymph N(GP), N+*p*, N+p^{Cho} at pH 7.0 and N+*p*RBL, N*p*RBL at both pH are isogenic to each other. Where as, in digestive juice, bivoltine lines express almost null at both pH, which strongly corroborated Doiras (1993) view though one very week α-amylase band was observed at pH 8.5.

Overall isozyme pattern in haemolymph and digestive juice at both pH depicted more number of β-amylase than α-amylase in all multivoltine lines including RBLs. Earlier, Day and Waterhouse (1953) stated the existence of β-amylase in haemolymph and mid-gut. The above findings is strongly corroborating with the present work and found that haemolymph of tropical multivoltine and bivoltine lines have both α and β-amylase but the digestive juice of bivoltine lines have no such amylase i.e., null for both. Before that, no such information is available on the existence of β-amylase in tropical silkworm. It is also observed that not a single native protein at both pH is common for expression of any band of amylase isozyme, *i.e.*, a totally different set of proteins are the amylase isozyme expresser at specific pH. Beside no specific amylase expresser native protein(s) was identified as a molecular factor associated with developed RBLs where transgression of cocoon shell weight was observed over receptor parents.

Further, it may be stated that RBLs provide better survival with an improvement in shell weight and filament length.

Acknowledgements

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