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[Short Communication]

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Isoelectric Focusing of the Hemolymph Proteins of the Silkworm, *Bombyx mori*

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Soluble proteins of silkworm hemolymph were investigated by means of isoelectric focusing (IEF). The protein spectra during ontogenesis of races and inter-races hybrids kept in Bulgaria was studied. A total of 51 protein bands in the hemolymph from fourth larval instar to imago were ascertained. Stage specific expression was established. The specific expression of some protein bands in the individual spectra manifest phenotype of gene determinate polymorphism (HP F, HP J, HP K, HP L, HP Q - in the zone with pH gradient 3.5 ~ 6.2 and HP K, HP L, HP N, HP P, HP T - in the zone with pH gradient 9.5 ~ 6.2). Breed specific expression was observed. On the basis of the obtained results, it was established that the investigated breeds are heterogeneous and the isoelectric focusing method is successful when specifying the inner-race and inter-race polymorphism in silkworm.

Key words: *Bombyx mori* L., Isoelectric focusing (IEF), Hemolymph proteins

Introduction

Isoelectric focusing (IEF) is a method with a high dividing capacity, exceeding many times the electrophoresis using agar, polyacrylamide and starch gels. This method is successfully used in the population-genetic and biochemical-genetic investigations. Even the slight distinctions in the net-charges, not detected by means of the conventional electrophoretic techniques, can be easily designated when using the isoelectric focusing. The proteins fractionating is made between two electrodes under the influence of the electric field and appears in the form of sharply focused bands in these sections of the gradient, where the protein loses its charge and is fixed over the relevant ampholines. Different electrophoretic methods (electrophoresis on paper, in agar, polyacrylamide and starch gels) had been used for analyzing different proteins and isozymes in silkworm (Philippovich and Allieva, 1966; Eguchi, 1968; Ostapenko *et al.*, 1975; Seong *et al.*, 1985, 1988; Lee *et al.*, 1985, 1992, 1994; Marcato *et al.*, 1990; Seong, 1991; Kawaguchi *et al.*, 1993; Banno *et al.*, 1993, 1994; Stoykova and Terzieva, 1998). The isoelectric focusing, however, is not a very popular method for investigation of *B. mori*. The protein spectra of races kept in Bulgaria had not studied for searching of inner-race and inter-race polymorphism and stage specific expression. These facts provoked us to provide this investigation on the protein spectrum of *B. mori* during ontogenesis using the IEF.

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Materials and Methods

By means of isoelectric focusing (IEF) 70 specimens (male and female) from different races designated as B517, T106, T108, M1, M2, UK17, UK18, UK19, UK20, P14, P15, Tashkent 16 as well as the F_1 inter-race hybrids P14 × P15 and P15 × P14 were investigated. The protein spectra in different ontogenetic stages (from fourth larval instar to imago) were analysed. Only individual samples were used.

Hemolymph was isolated by pricking one of the larval abdominal legs or the thorax of the pupa or the imago, collected in a tube containing a small amount of phenylthiourea. The hemolymph samples were mixed 1:1 with distillated water and left for 18 hrs at 4°C. They were anal-

ysed for total protein concentration by the method of Lowry *et al.* (1951) with bovine serum albumin as a standart as follows: fourth instar 2.4 μg/μl; fifth instar 1 – 2 day 5 μg/μl, fifth instar 5 – 7 day – 15 μg/μl; spinning period 18.2 μg/μl; pupae 1 – 2 day 15.8 μg/μl; pupae 5 – 6 day – 12 μg/μl; pupae 8 – 10 day – 9 μg/μl; imago 8 μg/μl.

Isoelectric focusing (IEF) was carried out in an LKB Multiphor apparatus on original Ampholine PAG plates (pH 3.5 ~ 9.5; 245 × 110 × 1 mm; Polyacrylamide gel T = 5% C = 3%; Ampholine conc. 2.2% w/v) at 1500 V, 50 mA, 30 W for 90 min at start position 4. The staining solution consisted of 460 mg Coomassie brilliant blue G 250 in 400 ml discolouring solution. The discolouring solution is a mixture of 500ml ethanol, 160 ml acetic acid and distilled water to 2l.

Results and Discussion

A total of 51 protein bands in the hemolymph (from fourth larval instar till imago) by means of IEF were ascertained (Fig. 1). The gel plate was provisionally separated of anode (pH gradient 3.5 ~ 6.2) and cathode (pH gradient 9.5 ~ 6.2) sections. The protein bands (HP hemolymph proteins) of the relevant plate section, were marked with letter symbols, in direction of gradient increase (in the 3.5 ~ 6.2 range) or gradient decrease (in the 9.5 ~ 6.2 range), *i.e.*, the outer-most one toward the start.

In the zone with pH 3.5 ~ 6.2 in direction of pH gradient increase they were visualized totally 23 protein bands, as follows: HP A, HP B, HP C, HP D, HP E, HP F1 and F2, HP G, HP H, HP I, HP J, HP K1 and K2, HP L, HP M, HP N, HP O, HP P, HP Q1 and Q2, HP R, HP S and HP T (Fig. 1). The HP A, B and C bands (pH 4.5 ~ 4.7) were detected in the hemolymph of all individuals and in all stages. In the zone with pH gradient 4.8 ~ 4.9 HP D and HP E with different intensity in the different ontogenetic stages were visualized. HP D was weak in the hemolymph of the fourth instar and the first half of the fifth instar. In the end of the last larval instar, the spinning period and the beginning of the pupa stage, HP D was distinct, and regarding imago it was again weak (Fig. 1). The band HP E was distinct and intensive from the fourth instar till the middle of the pupa stage. In the hemolymph spectrum of pupae 8 – 10 day and adults, this stripe was weaker. In the zone with pH gradient 5 ~ 5.8 was observed polymor-

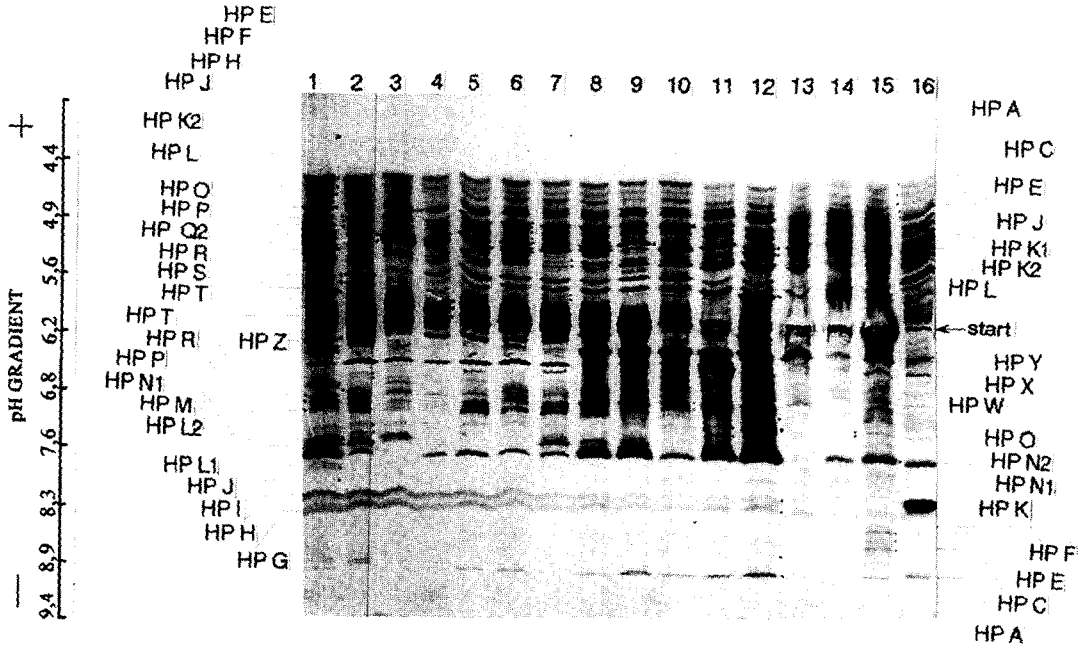

Fig. 1. Hemolymph protein IEF patterns of silkworm *B. mori* L.: lanes 1, race B517, 5th larval instar 4 – 5 day; lane 2, race UK20, 4th larval instar; lane 3, race P14, 4th larval instar; lane 4, race M2, 4th larval instar; lane 5, race P14, 4th larval instar; lane 6, race UK20, 4th larval instar; lane 7, race P15, 5th larval instar 2 – 3 day; lane 8, race T108, 5th larval instar 4 – 5 day; lane 9, race P15, 5th larval instar 7 day; lane 10, race UK19, spinning larvae; lane 11, race P15, pupae 1 – 3 day; lane 12, race M2, pupae 1 – 3 day; lane 13, race UK20, pupae 8 – 10 day; lane 14, hybrid P15 × P14, pupae 8 – 10 day; lane 15, hybrid P15 × P14, pupae 8 – 10 day; lane 16, hybrid P15 × P14, imago. Lanes 1 ~ 4, 9, 11 ~ 13 male specimens; 5 ~ 8, 10, 14 ~ 16 female specimens.

phism in the expression of some hemolymph protein fractions. In some individuals of the UK19, UK20 and M2 breeds, in HP F zone (Fig. 1, lane 10) were ascertained two bands (HP F_1 and F_2). In other individual spectra of the same, and all rest breeds, was visualized only one of them (Fig. 1, lanes 6 ~ 9, 11, 12). HP F_1 and F_2 were very closely situated, so they were distinguished with difficulties (Fig. 1, lane 10). The fraction HP H was distinct. Between HP F and HP H bands, was ascertained the weak HP G. The fraction HP I was also weak. The HP J band was intensive in some individuals spectrum of the investigated breeds (Fig. 1, lanes 3, 4, 6, 8, 9). In others spectrum HP J was slightly expressed (Fig. 1, lane 7) or lacking (Fig. 1, lanes 10, 12, 13). In some individuals from UK20, independently or in combination in pairs, were ascertained the bands HP K1 and HP K2 (Fig. 1, lanes 6, 16). In the individuals spectra from the rest breeds and hybrids, were visualized in this zone only one of the two fractions (Fig. 1, lanes 3 ~ 5, 7 ~ 13). We ascertained an intensive expression of HP L in some individual spectrum of the investigated breeds and hybrids (Fig. 1, lanes 1, 12), its weaker expression in others spectrum (Fig. 1, lanes 2, 6 ~ 9, 11, 13, 14), or its absence (Fig. 1, lanes 3 ~ 5, 10). The fractions HP M, HP N, HP O and HP P were visualized from the fourth larval instar to imago. The last two bands were slightly expressed in the spectrum of pupae 8 – 10 day (especially HP O). In the hemolymph of some individuals were observed the expression of two weak fractions HP Q1 and HP Q2 (Fig. 1, lane 7), but in the hemolymph of the others - only one of them with different intensity was expressed (Fig. 1, lanes 1, 3 ~ 6, 8, 10 ~ 12). In the spectra of some individuals they both were lacking at all (Fig. 1, lane 9). The fractions HP R, S and T (pH 5.9 ~ 6.2), were very closely situated, which makes their indication difficult. In the larval period and the spinning period, the bands from this zone were very intensive, and at the beginning of the pupa period we ascertained weakness till the total exhaustion of these proteins in the end of this period and in the imago stage. The fractions HP A, E, F, H, K, O, the bands of the zone HP R ~ T were clear and intensive in the hemolymph spectrum till the beginning of the pupae period, as well as the fractions HP J, L and Q. In the end of the pupae period and imago, the protein fractions in the anode section of the plates were with weaker expression.

In the zone with pH 9.5 ~ 6.2, in direction of decreasing the pH gradient were ascertained a total of 28 protein fractions, as follows: HP A, HP B, HP C, HP D, HP E, HP F, *HP G, HP H, HP I,* HP J, HP K, HP L1 and L2, HP M, HP N1 and N2, HP O, HP P, HP Q, HP R, HP S, HP T, HP U, HP V, HP W, HP X, HP Y and HP Z (Fig. 1). The fractions HP A, B and D (pH 9 ~ 8.7) were ascertained in all individual spectra. HP B was very weak, as well as HP F. We visualized the appearance of HP C and E in pupae 8 – 10 day. HP G was clear. HP H in the hemolymph of larvae and pupae was clear, too. In the imago period of the same place, were observed the presence of very intensive protein band (pH ~ 8.3), which manifested stage specificity (Fig. 1, lane16). The fractions HP I and J (pH 8.1 ~ 7.9) were slightly expressed and that is the reason they were difficult distinguishing. In some individual spectra from P14, P15, B517, T106, T108, M1, M2, UK17, UK18, UK19, UK20 and Tashkent 16, fraction HP K (pH ~7.8) was very intensive (Fig. 1, lanes 1, 8, 9, 11, 12). In others spectra this band was slighter (Fig. 1, lanes 2, 4 ~ 7, 10, 14 ~ 16) or lacking at all (Fig. 1, lane 13). In some individual spectra were observed fractions HP L1 and L2 (pH ~7.6) (Fig. 1, lane 1). In others spectra only one of them (Fig. 1, lanes 2, 7, 8) or the lack of the both was determined (Fig. 1, lanes 4 ~ 6, 10, 16). In the zone with pH gradient 7.4 ~ 6.7 was observed polymorphism in the expression of protein bands. The fraction HP M was weak (Fig. 1, lanes 2, 11) and in that reason difficult to be visualized in some samples. In some individual spectra were ascertained two slight bands HP N1 and N2 (Fig. 1, lane 14) and in others spectra only one of them (Fig. 1, lanes 1 ~ 3, 5, 6, 9 ~ 13, 15, 16). HP O and Q were distinct in the hemolymph of larvae and pupae 1 – 2 day, and more slightly expressed in the successive ontogenetic stages. Fraction HP P in the hemolymph of some individuals from B517 and UK19 was visualized (Fig. 1, lanes 1, 10). In the zone of HP R-S they were observed slight distinctions in the protein bands mobility and intensity. In some individual spectrum of the breeds B517, P14, UK20, UK19 we visualized fraction HP T (Fig. 1, lanes 1, 3). There was a weak expression of HP U and HP V in larvae, pupae and imago spectrum. The fraction HP W (pH ~6.6) was distinct in larvae and pupae 1 – 2 day, and weak in the successive stages. The bands HP X and Y were slightly expressed till the middle of the fifth instar, and intensive at the end of this instar, the spinning period and the pupae-turning stage. In the imago spectrum HP X and Y were weaker again. HP Z (pH ~6.3) was visualized in proximity of the start. It was more distinct till the middle of the fifth larval instar, and in the spectrum of pupae 8 – 10 day and imago spectrum HP Z was very slight. We observed weak distinctions in this band mobility in the separate breeds. At the end of the pupae period and in imago stage, the protein bands of the cathode section were weaker, as well as the protein bands of the anode section.

The observed stage specificity in the appearance of the hemolymph proteins indicates dynamics of the gene expression in the process of the ontogenesis. This confirms other authors (Seong, 1981; Krishnamurthy *et al.*,

1984; Raju and Krishnamurthy, 1995; Stoykova and Terzieva, 1998) earlier ascertained, by means of other electrophoretic methods. In the end of the individual development, the protein fractions were with weaker expression, probably as a result of the gradual exhaustion of some proteins.

The specific expression of some protein bands in the individual spectra manifest phenotype of gene determinate polymorphism (HP F, HP J, HP K, HP L, HP Q - in the zone with pH 3.5 ~ 6.2 and HP K, HP L, HP N, HP P, HP T - in the zone with pH 9.5 ~ 6.2). The codominant polymorphism in proteins of the silkworm hemolymph is described by Shimada *et al.* (1985) and Banno *et al.* (1994). Polymorphism with null alleles in proteins and non-specific esterases is earlier reported by He (1995), Stoykova *et al.* (1999) and Stoykova (2001).

Fractions HP F, HP K (pH 3.5 ~ 6.2), HP K, HP P, HP T and HP Z (pH 9.5 ~ 6.2) manifest breed specificity. The breed specificity in the expression of proteins and izoenzymes in *B. mori* is also described by other authors (Philippovich *et al.*, 1978; Xu and Song, 1987; Kanekatsu *et al.*, 1993).

On the basis of the obtained results by means of isoelectric focusing we can suppose that the investigated breeds raised in Bulgaria are heterogeneous, which probably is due to their different origin, as well as the carried out selection. The isoelectric focusing method is successful when specifying the inner-race and inter-race polymorphism. Further analyses of hemolymph proteins by IEF will be of interes

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