Int. J. Indust. Entomol. Vol. 14, No. 1, 2007, pp. 37~44

International Journal of
Industrial Entomology

Electrophoretic Analysis of Haemolymph Proteins during Silkworm (*Bombyx mori* L.) Ontogenesis

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(Received 8 February 2007; Accepted 27 February 2007)

A study was made of the haemolymph protein spectrum of mulberry silkworm (*Bombyx mori* L.) from the first larval instar to imago. Horizontal starch gel electrophoresis was used. Sixteen races and eight F1 interracial hybrids, raised in Bulgaria, were analyzed. During the ontogenesis, a total of 17 protein bands (15 cathodic and 2 anodic) were detected. Distinct dynamics in the haemolymph protein spectrum was observed, in result of different expression during the individual development associated with the processes of growth, histolysis and histogenesis. Based on the ontogenetic dynamics found, a correspondence was assumed between some proteins detected by us using the starch gel electrophoresis and major haemolymph proteins (SP1, SP2, MHPs and Vg) detected by other authors using the polyacrilamide gel electrophoresis. Intraracial and interracial polymorphism was observed in four protein zones. The effect of four polymorphic loci with codominant and null alleles was suggested.

Key words: *Bombyx mori* L., Haemolymph proteins, Ontogenesis, Starch gel electrophoresis

Introduction

Different authors established a different number of ptotein bands in the haemolymph of mulberry silkworm *Bombyx mori* L. These differences resulted from the use of different electrophoretic techniques for analysis, as well as from the study of races with different origin and geographic distribution. For example, using a polyacrylamide gel electrophoresis (PAGE), Doira (1968) detected more than 20 protein fractions in the haemolymph of 5th larval instar and pupae, and Shtegoleva and Filipovich (1970) visualized from 17 to 25 protein bands. Kovalevskaya and Filipovich (1970) described from 9 to 21 protein bands in 3rd instar larvae to imago. Some authors reported also the presence of polymorphism at specific protein loci (Gamo and Othsuka, 1980; Lee *et al.*, 1985; Banno *et al.*, 1994). The so-called major proteins were mainly studied. No analyses were made on the zones where less expressed protein bands were visualized. Most studies were conducted only at specific ontogenetic stages.

The protein polymorphism in the *B. mori* L. races and hybrids reared in Bulgaria has been poorly studied (Abadjieva *et al.*, 1978; Abadjieva and Tanev, 1980, 1983; Abadjieva and Malinova, 1998). No analyses were done on the full dynamics of electrophoretic spectrum of the soluble haemolymph proteins from the 1st larval instar to imago. All this motivated the present study.

Materials and Methods

By means of Starch gel electrophoresis 300 specimens from different races designated as P14, P15, 19, 20, B517, T106, T108, M1, M2, UK17, UK18, UK19, UK20, Tashkent12, Tashkent15 and Tashkent16 as well as the interracial F1 hybrids P14×P15, P15×P14, M1×M2, M2×M1, UK17×UK19, UK19×UK17, UK18×UK20 and UK20×UK18 were studied. The races P14, P15, 19 and 20 were obtained in Bulgaria and the rest were introduced. The race B517 is polyvoltine, while all others are monovoltine. Specimens were selected from different families of each race including reciprocal hybrids. Haemolymph protein spectra were analyzed at various stages of silkworm development (larva - 1st, 2nd, 3rd, 4th and 5th

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Table 1. Total protein concentration of haemolymph samples tested in different ontogenetic stages

Stage of ontogenesis	Total protein concentration for tested samples (μg/μl)	
	individual samples	mixed samples
1st larval instar	0.7	5.4
2nd larval instar	1.1	9.7
3rd larval instar	1.6	12.4
4th larval instar	2.4	16.2
5th larval instar 1-4 day	5.0	-
5th larval instar 5-7 day	15	-
spinning larvae	18.2	-
pupae 1-2 day	15.8	-
pupae 5-6 day	12	-
pupae 8-10 day	9.0	-
imago	8.0	-

instar, spinning larva, pupa - 1-2 day, 5-6 day, 8-10 day and imago). In the 1st, 2nd, 3rd and 4th larval instars we tested individual samples (haemolymph isolated from a single specimen) as well as mixed samples (haemolymph isolated from several specimens) per race and hybrid. At all other ontogenetic stages samples were taken from a single specimen only. The sex at the stages from 4th larval instar to imago was also recorded.

Haemolymph was isolated by pricking one of the larval abdominal legs or the thorax of the pupa or the imago and collected in a tube containing a small amount of phenylthiourea. The haemolymph samples were mixed 1:1 with distillated water. They were analysed for total protein concentration by the method of Lowry *et al.* (1951) with bovine serum albumin as a standart (Table 1).

The electrophoretic separation involved using 13% Starch horizontal gels at 2.5 mA/cm for 5 hours, 0.05 M tris-EDTA-borate gel buffer at pH 8.1 and 0.3 M sodium-borate electrode buffer at pH 8.9 (Smithies, 1955, modified by Dobrovolov, 1973). Soluble proteins were displayed by staining with Amidoschwartz 10B for 10-15 min. The starch plates were differentiated in 5% acetic acid.

Results and Discussion

During ontogenesis (from the first larval instar to imago) we detected the expression of a total of 17 protein bands in the haemolymph (Hemolymph Proteins - HP) marked

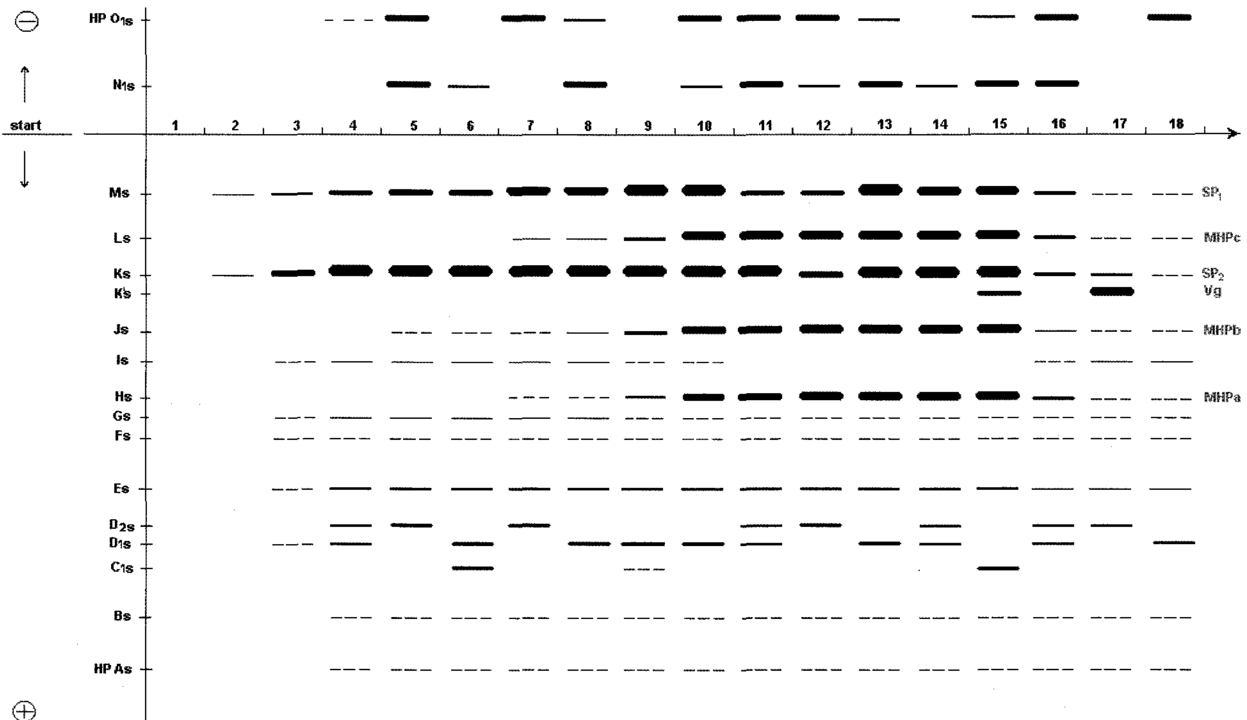

Fig. 1. Scheme of protein spectra of silkworm (*Bombyx mori* L.) haemolymph during ontogenesis - 13% starch gel electrophoresis: 1st and 2nd larval instars (1, 2); 3rd larval instar (3, 4); 4th larval instar (5, 6); 5th larval instar 1-4 day (7, 8); 5th larval instar 5 day (9); 5th larval instar 6-8 day (10, 11); spinning larvae (12, 13); pupae 1-2 day (14); pupae 5-6 day (15); pupae 8-10 day (16); adults (17, 18). Lines 10, 13-15 and 17-spectra of female specimens; 11, 12, 16 and 18-spectra of male specimens. Lines 2, 4-mixed probes of haemolymph; 1, 3, 5-18-individual probes of haemolymph. Lines 6, 9 and 15-spectrum of polyvoltine race B517.

from the anode to the cathode in the following way: HP As, HP Bs, HP C_1s, HP D_1s, HP D_2s, HP Es, HP Fs, HP Gs, HP Hs, HP Is, HP Js, HP K's, HP Ks, HP Ls, HP Ms, HP N_1s and HP O_1s (Fig. 1).

In mixed haemolymph samples of 1st and 2nd larval instar, fractions HP Ks and HP Ms were detected (Fig. 1). No protein bands were found in the spectra of individual samples, which were associated with their low protein concentration at these ontogenetic stages.

In mixed haemolymph samples of 3rd larval instar, the following anodic fractions were observed: HP As, HP Bs, HP C_1s, HP Ds (D_1s and D_2s), HP Es, HP Fs, HP Gs, HP Is, HP Ks and HP Ms (Fig. 1 and 2). Most intensive in this ontogenetic period were bands HP Ks and HP Ms. In the cathodic part of the gel, we determined also HP O_1s - weak. In individual haemolymph samples, distinct HP Ks and HP Ms were detected, as well as very weak traces of the bands in zones HP C_1s - HP Is and HP O_1s. Fraction HP C_1s was detected only in some individuals of the polyvoltine race B517. In zone HP Ds, two protein bands - HP D_1s and HP D_2s, were ascertained, expressed either separately or in combination in the spectra of different individuals.

In mixed and individual samples of 4th larval instar, the

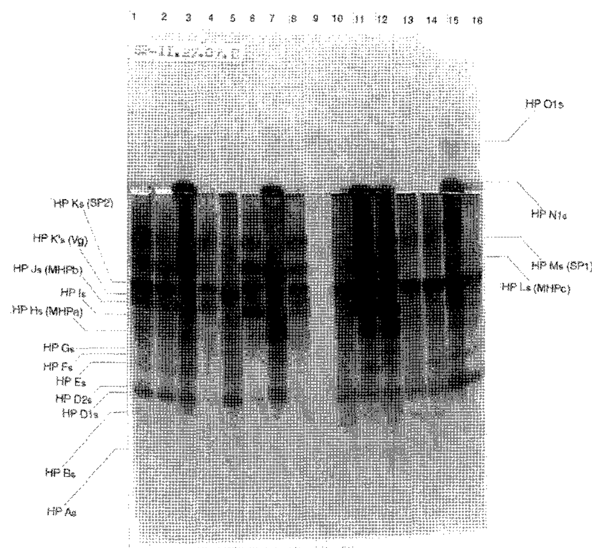

Fig. 3. Protein spectra of silkworm (*Bombyx mori* L.) haemolymph - 13% starch gel electrophoresis: 4th larval instar (13, 14), 5th larval instar 1 day (5, 10) and 6-7 day (1, 2, 15, 16), spinning larva (11), pupae 2 day (6, 7, 8, 12) and 7 day (3, 4), imago (9). Races B517 (1-4), T108 (5-9), M1 (10-12) and P15 (13-16). Samples 1, 3-6, 8, 11-15-female specimens; 2, 7, 9, 10, 16-male specimens.

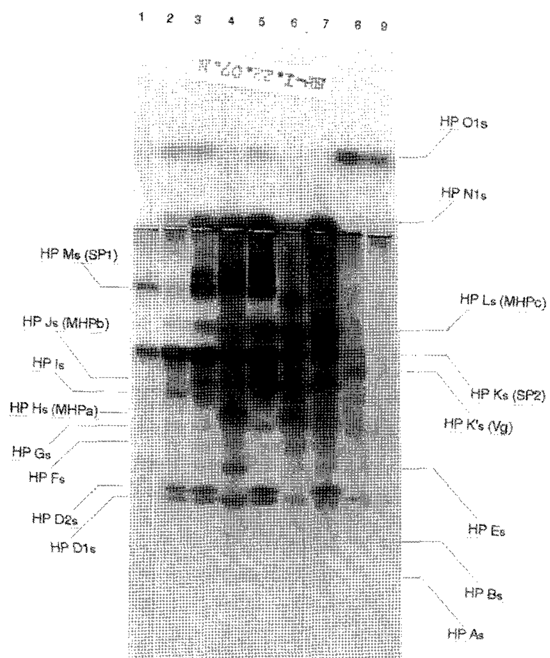

Fig. 2. Protein spectra of silkworm (*Bombyx mori* L.) haemolymph-13% starch gel electrophoresis: 3rd larval instar (1), 5th larval instar 4 day (2, 3, 5) and 7 day (4), pupae 1-2 day (6) and 5 -6 day (7), imago (8, 9). Races T108 (1, 6), M1 (4), P14 (7), P15 (8, 9) and hybrids P14×P15 (2) and P15×P14 (3, 5). Samples 4, 7, 9-male specimens and 2, 3, 5, 6, 8-female specimens; 1-mixed probe, 2-9-individual probes.

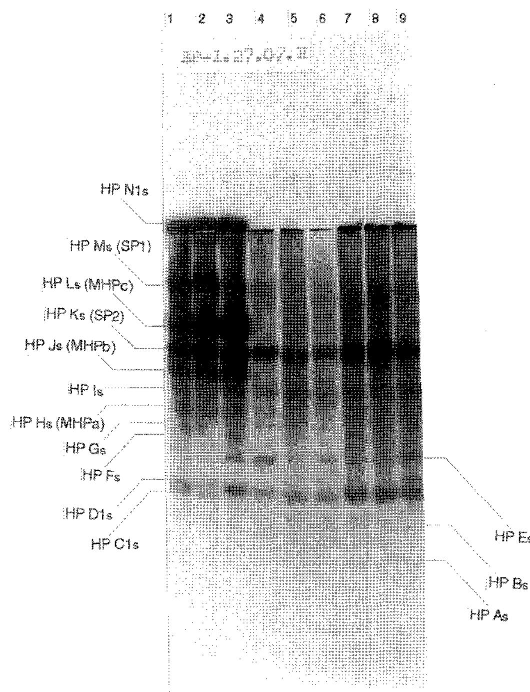

Fig. 4. Protein spectra of silkworm (*Bombyx mori* L.) haemolymph - 13% starch gel electrophoresis: 4th larval instar (7-9), 5th larval instar 1 day (4-6) and 7 day (1-3). Races B517 (1-3), T108 (4-6) and M1 (7-9) - female specimens.

occurrence of HP Js and HP N_1s was detected (Fig. 1 and 3).

During the first half of the 5th larval instar (days 1-4), the following protein bands were ascertained: HP As and HP Bs - weak; HP C_1s - only in some individuals of the race B517; HP D_1s and D_2s - alone or in combination; HP Es; HP Fs; HP Gs; HP Is; HP Js; HP Ks; HP Ms; HP N_1s and HP O_1s. At the start of this age, the appearance of two very weak bands was observed - HP Hs and HP Ls. HP Js was also slightly expressed (Fig. 1, 3 and 4). During the second half of the 5th instar (days 5-8), we established some weakening of HP Gs and increasing in the intensities of HP Hs, Js and Ls (Fig. 1, 2, 3 and 4). HP Ks and Ms were also very intensive. Fraction HP Is weakened and disappeared till the end of this period. The spectra of male individuals during the second half of the 5th instar, showed weaker expression of HP Ms (Fig. 1 and 3).

In the haemolymph of spinning larvae (Fig. 1 and 3), weaker expression of band HP Ks was detected in the male individuals. The sex differences, observed in the expression of HP Ks and HP Ms during the spinning period and the second half of the last larval age, remained till the end of the individual development.

During the pupal period (pupae of 1-2, 5-6 days), in the anodic part of the gel the following protein bands were detected: HP As; Bs; C_1s; D_1s and D_2s; Es; Fs; Gs; Hs; Js; Ks; Ls and Ms. In the cathodic part, the bands HP N_1s and O_1s were found (Fig. 1, 2 and 3). The most intensive in that period of individual development were HP Hs, Js, Ks, Ls and Ms. HP N_1s and HP O_1s proteins were expressed with different intensity only in the spectra of some pupae. From the beginning of the pupal stage to the end of the individual development, band HP K's was detected in the spectra of some females. This band was completely absent in the spectra of males. At the end of the pupal period (pupae of 8-10 days), a decreased intensity of the protein fractions and the reappearance of band HP Is were established (Fig. 1).

In the adult spectrum, we determined: HP As; Bs; C_1s (only in individuals of the race B517); Ds (D_1s and D_2s); Es; Fs; Gs; Hs; Is; Js; K's (only in female individuals); Ks; Ls; Ms; N_1s and O_1s. The bands HP Hs, HP Js, HP Ls and HP Ms were very weak, which made them difficult to document in some samples. All the other fractions with anodic mobility, except HP Is - in both sexes, and HP Ks and HP K's - in the females, were also weaker as compared to the pupal period (Fig. 1 and 2). At the imago stage, an increased expression of HP K's was observed (Fig. 2).

In our previous investigations (Stoykova and Terzieva, 1998) we reported the presence of more protein bands than the above described ones. In the present work, we analyzed more individuals, including other races, too, and found that some of the bands, observed earlier, were expressed either as slight traces or were completely absent

Table 2. Race specific expression of silkworm haemolymph proteins from polymorphic zones: (+) expression, (−) absent expression.

Races	Protein zone				
	HP Cs	HP Ds		HP Ns	HP Os
		bands			
	band HP C_1s	HP D_1s	HP D_2s	band HP N_1s	band HP O_1s
P14	−	−	■	+	+
P15	−	■	−	+	+
19	−	+	+	■	+
20	−	+	+	−	−
B517	+	■	−	+	+
T106	−	■	−	+	+
T108	−	+	+	+	+
M1	−	■	−	+	+
M2	−	■	−	+	+
UK17	−	■	−	+	+
UK18	−	+	+	+	+
UK19	−	■	−	+	+
UK20	−	■	−	+	+
Tashkent 12	−	■	−	−	+
Tashkent 15	−	■	−	−	+
Tashkent 16	−	■	−	+	+
Hybrids					
P14×P15	−	■	■	+	+
P15×P14	−	■	■	+	+
M1×M2	−	■	−	−	+
M2×M1	−	■	−	−	+
UK17×UK19	−	■	−	−	+
UK19×UK17	−	■	−	−	+
UK18×UK20	−	+	+	−	+
UK20×UK18	−	■	−	−	+

■ The band was expressed in the haemolymph of all tested individuals from the race or hybrid.

[+] The band was expressed only in the haemolymph of some tested individuals from the race or hybrid.

from the spectra of most individuals tested, which was the reason for not commenting on them.

Analyzing the individual haemolymph protein spectrum, we detected polymorphism and race specificity in the expression of bands from zones HP Cs, HP Ds, HP Ns and HP Os, i.e. (Table 2):

- Fraction HP C_1s was expressed only in the haemolymph of some individuals of the polyvoltine race B517. The absence of this band from the spectra of other

individuals, as well as its different intensity allowed our assumption for a biallelic polymorphism at the specific locus with a null allele. In a homozygotic state, this conditioned the lack of a protein band in zone HP Cs, and in a heterozygotic one-the expression of a weaker band. HP C_1s was not detected in the monovoltine races and their hybrids, which was probably due to the null allele homozygosity.

- Fractions HP D_1s and HP D_2s were expressed either alone or in combination in the spectra of different individuals of races 19, 20, T108, UK18 and the F1 hybrid UK18 × UK20. This allowed us to assume a polymorphism with two codominant alleles at the specific locus. In the spectrum of race P14, we determined only band HP D_2s (probably homozygotes for one allele). In races P15, B517, T106, M1, M2, UK17, UK19, UK20, Tashkent12, Tashkent15, Tashkent16 and the hybrids M1 × M2, M2 × M1, UK17 × UK19, UK19 × UK17 and UK20 × UK18, only HP D_1s was observed (probably homozygotes for the other allele). In the haemolymph of all analyzed hybrids of P14 × P15 and P15 × P14, we detected the simultaneous presence of both bands (probably heterozygotes).
- Fraction HP N_1s was expressed in all individuals of race 19. This band was not expressed either in the spectra of races 20, Tashkent12, Tashkent15 or in the hybrids M1 × M2, M2 × M1, UK17 × UK19, UK19 × UK17, UK18 × UK20 and UK20 × UK18. Amongst all other races and interracial hybrids, HP N_1s was present in the haemolymph of some individuals and absent from the haemolymph of other individuals. In the spectrum of race 20, no fraction HP O_1s was detected, either. This band was expressed with different intensity in the haemolymph of some individuals from the other races and their hybrids. The expression of fractions HP N_1s and HP O_1s allowed our assumption that each of these two protein bands with cathodic mobility was probably controlled by a polymorphic locus with a null allele. We assumed that the absence of HP N_1s and HP O_1s from the spectrum of race 20 was in result of homozygosity for the null alleles of both polymorphic loci. We supposed simultaneous polymorphism at both loci in races P14, P15, B517, T106, T108, M1, M2, UK17, UK18, UK19, UK20, Tashkent16 and the hybrids P14 × P15 and P15 × P14 (Table 2).

In our previous studies (Staykova *et al.*, 2004), using Isoelectric focusing (IEF) in the haemolymph spectrum, we detected fraction HP K (pH-7.8), which was very intensive in some individuals of races P14, P15, B517, T106, T108, M1, M2, UK17, UK18, UK19, UK20, Tashkent16

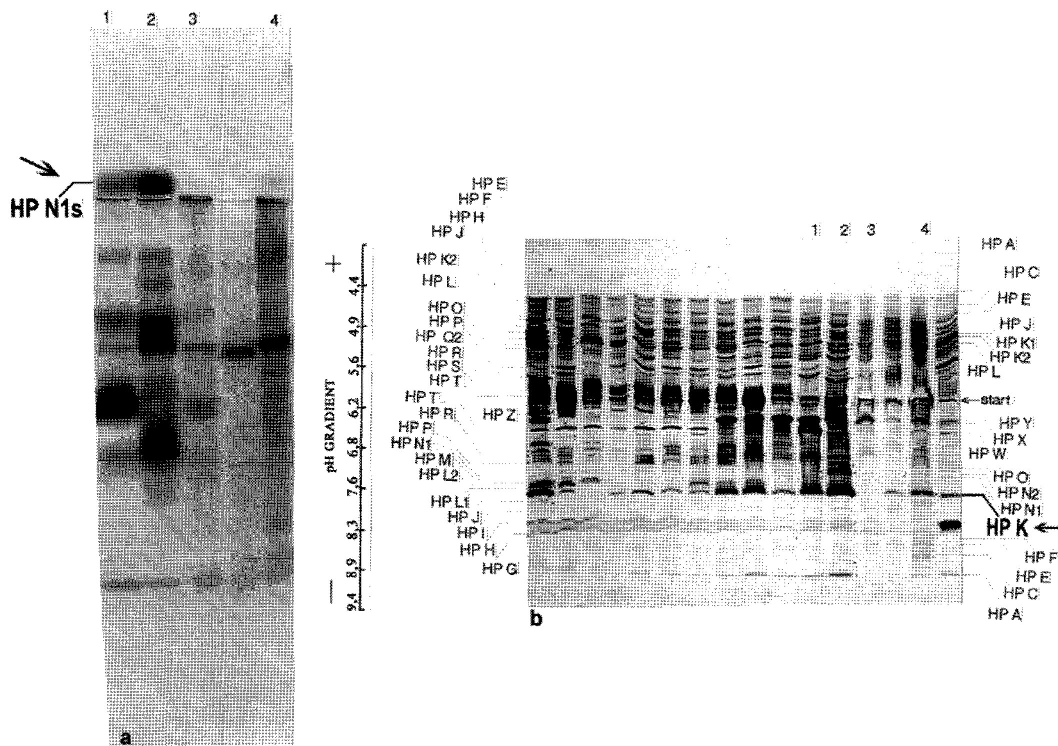

Fig. 5. Protein spectra of silkworm (*Bombyx mori* L.) haemolymph - pupae 1-3 day (1, 2) and 8-10 day (3, 4):
(a) 13% Starch gel electrophoresis;
(b) Isoelectric focusing in ampholine PAG plates (pH 3.5-9.5) at start position 4.
Races P15 (1), M2 (2), UK20 (3) and hybrid P15 × P14 (4). Samples NO.1-3 - male specimens; 4 - female specimen.

and the hybrids P14×P15 and P15×P14, and weaker or completely absent - in others. HP K was not found in the spectra of races 20, Tashkent12 and Tashkent15. Based on the race specificity observed in the expression of bands HP N_1s and HP K, detected through Starch gel electrophoresis and Isoelectric focusing, respectively, we could assume that band HP N_1s corresponded to HP K. The comparison of the results obtained in the parallel study of the same haemolymph samples by using both electrophoretic techniques, confirmed this correspondence (Fig. 5). The results obtained through Starch gel electrophoresis confirmed also the genetically determined polymorphism with a null allele, ascertained through IEF (Staykova *et al.*, 2004). We think that the absence of this specific protein from the haemolymph of some individuals, detected by the two electrophoretic techniques, was associated with homozygosity for the null allele.

The polymorphism found in zones HP Ds and HP Ns confirmed that determined by us earlier in zones SP 5-6 and SP22 (Stoykova and Terzieva, 1998).

Comparing our results for the ontogenetic dynamics in the haemolymph protein expression with the results obtained by other authors, the following analogies could be made:

- The stage-specific expression of HP Hs, HP Js and HP Ls conditioned our assumption that these protein bands corresponded to MHPa, MHPb and MHPc proteins, described by Seong *et al.* (1985) with molecular mass of 30 kDa. Li and Huang (1991) reported that the concentration of MHPs proteins increased in the 5th instar, which corresponded to the increased expression of bands HP Hs, HP Js and HP Ls observed by us in this stage. The weak expression of the HP Hs, HP Js and HP Ls at the end of the pupal period coincided with the quantitative changes in the group of the 30 kDa haemolymph proteins during pupation, reported by Seong *et al.* (1985) and Kawaguchi *et al.* (1996).
- The sex differences that we observed in the expression of fractions HP Ks and HP Ms during ontogenesis, conditioned our assumption that these bands corresponded to the storage proteins SP2 and SP1, described by Tojo *et al.* (1980). Tomino (1985), Tomino and Izumi (1985) reported that the sex-dependent expression of SP1 was genetically determined and it was observed during the larval period only in the 5th instar. This coincided with the "female" specificity of SP1, described also by other authors (Nagata and Kobayashi, 1990; Banno *et al.*, 1993; Kawaguchi *et al.*, 1994, 1997). Shimada *et al.* (1985) detected by PAGE three electrophoretic variants of the storage protein SP1 and two - of the storage protein SP2. They assumed biallelic codominant polymorphism at the SP2-coding locus and triallelic codominant polymorphism at the locus coding for SP1. Kawaguchi *et al.* (1986) confirmed the polymorphism at the one locus. No polymorphism was found by us in the storage protein zone of the tested silkworm races and their hybrids. In our opinion, this was due to the monomorphic races reared in our country at the respective loci. The occurrence of fraction HP K's in the haemolymph of female pupae and the presence of this band only in the spectra of females till the end of their individual development, conditioned our assumption that HP K's corresponded to the vitellogenin (Vg), described by Tojo *et al.* (1980), Ogawa and Tojo (1981), Seong *et al.* (1985). Tojo *et al.* (1980) assumed that SP2 was used for vitellogenin synthesis. Ogawa and Tojo (1981) observed that the Vg amount in the female individuals at the beginning of the pupal period was low but it gradually increased till the end of this stage. This corresponded to the increase of the HP K's intensity, detected by us. Vitellogenin was produced by the storage protein SP2 through its modification in the fat body (Lee *et al.*, 1994). By the haemolymph, this protein was transported into the maturing ova and used therein for Vtn synthesis (Fujikawa *et al.*, 1995). According to Kawaguchi *et al.* (1996, 1998) the vitellogenin synthesis in the fat body of *B. mori* probably started as early as during the spinning period.

On the basis of our results and their interpretation we could say that using horizontal starch gel electrophoresis a total of 17 protein bands were detected in the mulberry silkworm haemolymph from the first larval instar to imago. The specific expression of the protein fractions in four zones showed the phenotypic pattern of genetically determined polymorphism. The interracial differences found in the polymorphic protein zones were probably associated with the different origin, the different ecologo-genetical differentiation and the specificity of the breeding process of the different races. During the individual development of silkworms, we observed some changes in the haemolymph protein spectrum, which were considered a result of different expression during ontogenesis, associated with the processes of growth, histolysis and histogenesis. Thus, we confirmed the results obtained by other authors using other electrophoretic techniques (Kovalevskaya and Filipovich, 1970; Tojo *et al.*, 1980; Tomino, 1985; Tomino and Izumi, 1985; Nagata and Kobayashi, 1990; Banno *et al.*, 1993, 1994; Kawaguchi *et al.*, 1994; Kawaguchi *et al.*, 1997a, 1997b; Krishnan *et al.*, 1999). In the zones HP Ks and HP Ms we observed permanent expression from the first larval age to imago. The expression of all other protein bands was specific for the specific stages of the silkworm individual development.

Acknowlegment

This study had been assisted by "Fund for Scientific

Researches" at Ministry of Education and Science-contract VU-AN-2/2005.

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