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Evolution of Hemocyanin Subunits in Mygalomorph Spiders: Distribution of Hemocyanin Subunits and Higher Classification of the Mygalomorphae

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ABSTRACT—Mygalomorph spider's hemocyanins examined contain monomer subunits which are the component parts of hemocyanin hexamers and dimer subunits which bond two hexamers together. We determined the N-terminal amino acid sequences of twenty-two monomer subunits and ten constituent monomers of the dimer subunits prepared from hemolymph of seven mygalomorph spiders. We classified these subunits into eight groups based on the sequence comparison. From the distribution of monomer subunits in mygalomorph spiders, it is evident that duplications and losses of monomer subunits occurred frequently. This is the reason why we cannot detect an orthologous monomer subunit derived from a common ancestral sequence in the seven spiders. These monomer subunits were found in either Atypoidea or Avicularioidea. On the other hand, the dimer subunits having similar N-terminal sequences were found widely in both mygalomorph spider groups, showing that the dimer subunits are more conservative than monomer subunits for their function different from monomer subunits.

INTRODUCTION

Hemocyanins are the blue respiratory pigments found in the blood of many arthropods and are organized as hexamers of monomer subunit chains or multihexamers of these hexamers. The monomer subunits associate noncovalently in most cases, although a few hemocyanins contain one or more disulfide-linked dimers. The heterogeneity of the monomer subunits of arthropodan hemocyanins was demonstrated by means of polyacrylamide gel electrophoresis, but each of these subunits has a mass of about 75KDa and carries one oxygen-binding site, suggesting that all are clearly derived from a common ancestral protein (Van Holde and Miller, 1995). In hemocyanins of a crayfish and some spiders, it became clear that their hemocyanins contain the disulfide-linked dimers and the dimer has unique functions as a "linker" molecule, which bonds hexamers together (Markl, 1980; Van Bruggen *et al*., 1980; Markl *et al*., 1981).

A mygalomorph spider, *Aphonopelma* (as *Eurypelma*) *californicum* is one of the animals whose hemocyanins have been researched in detail. It was clearly demonstrated that its hemocyanin is composed of seven types of subunits (a–g) and the heterodimer bc, which functions as a "linker" molecule (Van Bruggen *et al*., 1980; Markl *et al*., 1981) and shows phenoloxidase activity (Decker and Rimke, 1998). The complete amino acid sequences of monomer subunits a (Voit and Feldmaier-Fuchs, 1990), d (Schartau *et al*., 1983) and e (Schneider *et al*., 1983) and 93% of the sequence of a subunit b (Sonner *et al*., 1990), which made up the heterodimer with a subunit c, were analyzed. Using the complete amino acid sequence data, phylogeny inference programs produced branching patterns for the hemocyanin subunits from *A. californicum* and horseshoe crabs, *Limulus polyphemus* and *Tachypleus tridentatus* (Beintema *et al*., 1994; Burmester and Scheller, 1996). However, the patterns could not give any evolutionary relation between animals having these subunits, while they showed evolutionary trees of hemocyanin monomer subunits.

In recent studies on the higher classification of the mygalomorph spiders, Raven (1985) divided the mygalomorph spiders into two groups, Tuberculotae and Fornicephalae, and Goloboff (1993), based on cladistic analysis of the relationships of mygalomorph spider families, resurrected the group Atypoidea, which was restricted to Antrodiaetidae and Atypidae, and the group Avicularioidea including the rest of the Mygalomorphae. They supported the monophyly of Antrodiaetidae and Atypidae, but did not reach the consensus of interpreting the relationships within the mygalomorphs as a whole.

On the other hand, from comparison of N-terminal amino acid sequences of hemocyanin subunits, the orthologous subunits which are available for making evolutionary tree of animals were found in horseshoe crabs (Sugita and Murayama, 1998), scorpions (Sugita *et al*., 1999) and araneomorph spiders (Takasu and Sugita, 1997), respectively. Furthermore,

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they could discuss origins and duplications of the hemocyanin subunits during the evolution of animals having these subunits. The N-terminal sequence analysis is regarded as a good method to step foot in the research of the evolution of hemocyanin subunits.

In this paper, as the first step to study the evolution of hemocyanin subunits in mygalomorph spiders, we analyzed the N-terminal amino acid sequences of twenty-two monomer subunits and ten constituent monomers of the dimer subunits from seven mygalomorph spiders and classified them into eight groups. Furthermore, based on the distribution of the subunits in the Mygalomorphae, we discuss the evolution of hemocyanin monomer and dimer subunits and the higher classification of the mygalomorph spiders.

MATERIALS AND METHODS

The antrodiaetids, *Antrodiaetus roretzi* and *Antrodiaetus yesoensis* were collected at Mt. Tsukuba, Ibaraki Prefecture and in Sapporo city, Hokkaido Prefecture, respectively. The atypids, *Atypus karschi* were collected in Tsukuba city, Ibaraki Prefecture. The ctenizids, *Latouchia typica* and *Ummidia fragaria* were collected in Zushi city, Kanagawa Prefecture and at Mt. Tsukuba, Ibaraki Prefecture, respectively. The hexathelids, *Macrothele yaginumai* and *Macrothele gigas* were collected in Iriomotejima island, Okinawa Prefecture and Ishigakijima island, Okinawa Prefecture, respectively.

The legs of a spider were cut by a razor-edge and the hemolymph bled from sections of the legs was sucked into a microsyringe. To dissociate native hemocyanin molecules into the component monomer subunits, the hemolymph was mixed with a triple Tris-EDTA buffer containing 67mM Tris, 13mM EDTA (pH 8.9) and stood overnight at 4°C. After removing sediments by centrifugation at 13,000 rpm for 5 min, the supernatant was kept with an equal volume of glycerin at –20°C as a hemocyanin sample.

Polyacrylamide disc and slab gels at pH 8.9 were prepared according to the method of Davis (1964). Electrophoresis of hemocyanin samples was carried out using Davis's tank buffer without dilution. After electrophoresis, gels were stained for detecting protein with 0.6% Coomassie brilliant blue (CBB) in 45.5% ethanol and 9.2% acetic acid, and for detecting copper with 0.08% rubeanic acid in 41.7% methanol and 16.7% acetic acid according to the method of Horn and Kerr (1969). In order to distinguish hemocyanin dimer subunits from monomer subunits, acrylamide concentration was varied from 5.25 to 9.75% by changing the volume ratio of acrylamide solution to water (Sugita and Sekiguchi, 1975).

The hemocyanin subunits in a disc gel were re-electrophoresed into a slab gel to separate hemocyanin subunits completely (Takasu and Sugita, 1997) and the subunits in the slab gel were transferred to a polyvinylidene difluoride (PVDF) membrane.

When sufficient amount of subunit protein for sequence analysis was not prepared, the subunit protein was electrophoretically concentrated as stated below. The subunit protein spot in several slab gels stained by CBB was cut out and the gel pieces were homogenized together in a little SDS-sample buffer that contained 25mM Tris, 0.1% SDS, 0.1% 2-mercaptoethanol (pH 6.8). After dialyzing overnight at 4°C against the SDS-sample buffer, the homogenized gel was kept at –20°C as a SDS-sample. The SDS slab gel was prepared according to the method of Laemmli (1970) and the proteins in the SDS-samples were concentrated by being re-electrophoresed into the SDS slab gel. The concentrated proteins in the SDS slab gel were transferred to a PVDF membrane.

The dimer band in the gel was cut out, homogenized and dialyzed against the SDS-sample buffer. The SDS-samples were re-electrophoresed into the SDS slab gel to separate the constituent monomer peptides of the dimers. The peptides electrophoresed into the SDS slab gel were transferred to a PVDF membrane.

The PVDF membrane was activated for 1 min in 100% methanol

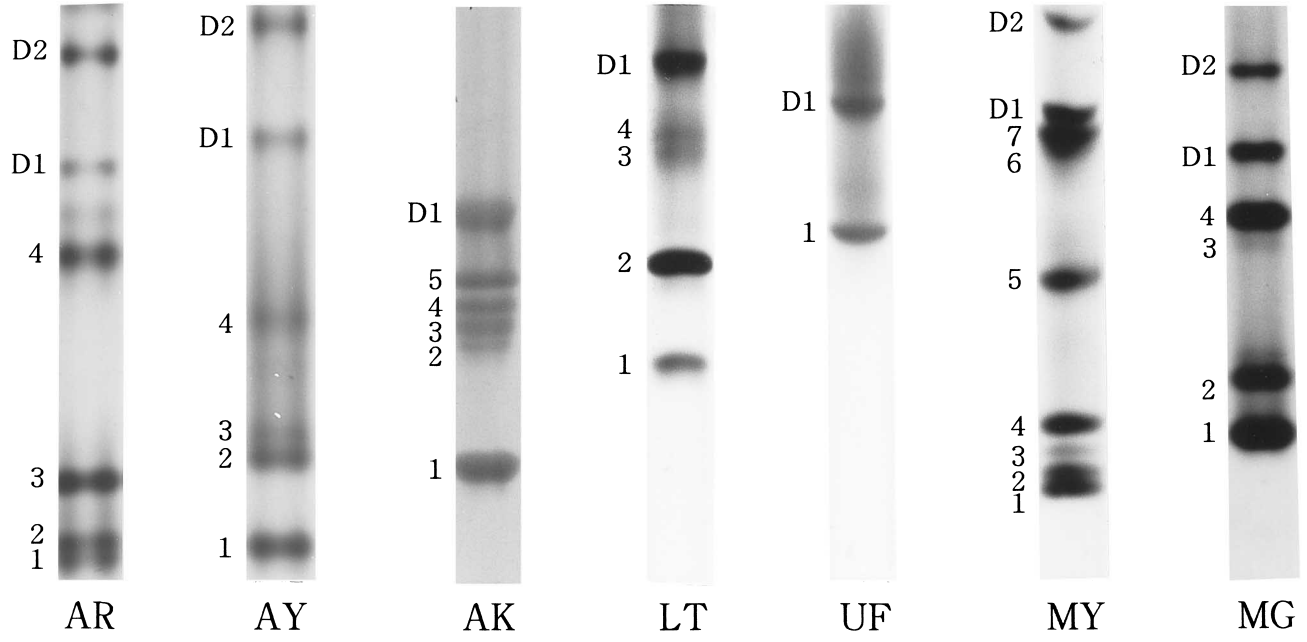

Fig. 1. Native PAGE banding patterns of hemocyanin subunits of seven mygalomorph spiders. AR, AY, AK, LT, UF, MY and MG show hemocyanin subunits of *A. roretzi*, *A. yesoensis*, *A. karschi*, *L. typica*, *U. fragaria*, *M. yaginumai* and *M. gigas*, respectively. The 7.5% acrylamide gels were prepared for electrophoresis of hemocyanins of the spiders except *M. yaginumai.* The 8.8% acrylamide gel were prepared for hemocyanin of *M. yaginumai*, because monomer subunits MY6 and MY7 overlapped with dimer subunit MY-D1 in the 7.5% acrylamide gel. The hemocyanin monomer bands were numbered from the bottom to the top of the gels, and the hemocyanin dimer bands were denoted D1 and D2.

and soaked in the transfer buffer. The transfer buffer contained 25mM Tris, 192mM glycine, 4% methanol, and 0.02% dodecyl sodium sulfate (pH 8.3). Electrophoretic transfer was carried out for 7 hr at 1.0 mA/cm^2 in a blotting apparatus (Towbin *et al.*, 1979).

The portion of PVDF membrane containing the hemocyanin subunit was cut out and mounted in the reaction chamber of a protein sequencer. The N-terminal sequences analysis was performed with Applied Biosystems model 447A or Procise 492 gas phase sequencer.

RESULTS

In order to distinguish the hemocyanin subunits from other proteins, the mygalomorph hemocyanin samples were electrophoresed into acrylamide gels and the gels were stained to detect the protein and the copper. The hemocyanin monomer bands were numbered from the bottom to the top of the gel (Fig. 1). The hemocyanins of *A. roretzi*, *A. yesoensis*, *A. karschi*, *L. typica*, *U. fragaria*, *M. yaginumai* and *M. gigas* showed four (AR1–4), four (AY1–4), five (AK1–5), four (LT1–4), one (UF1), seven (MY1–7), and four (MG1–4) monomer bands, respectively. The native PAGE banding pattern of the hemocyanin monomer subunits of *A. karschi*, *M. yaginumai* and *M. gigas* showed the polymorphism which was caused by allelic products, respectively. The banding patterns of the three hemocyanins shown in Fig. 1 were the most usual in each species, respectively. Furthermore, the protein bands of the three hemocyanins shown in Fig. 1 were found in all indi-

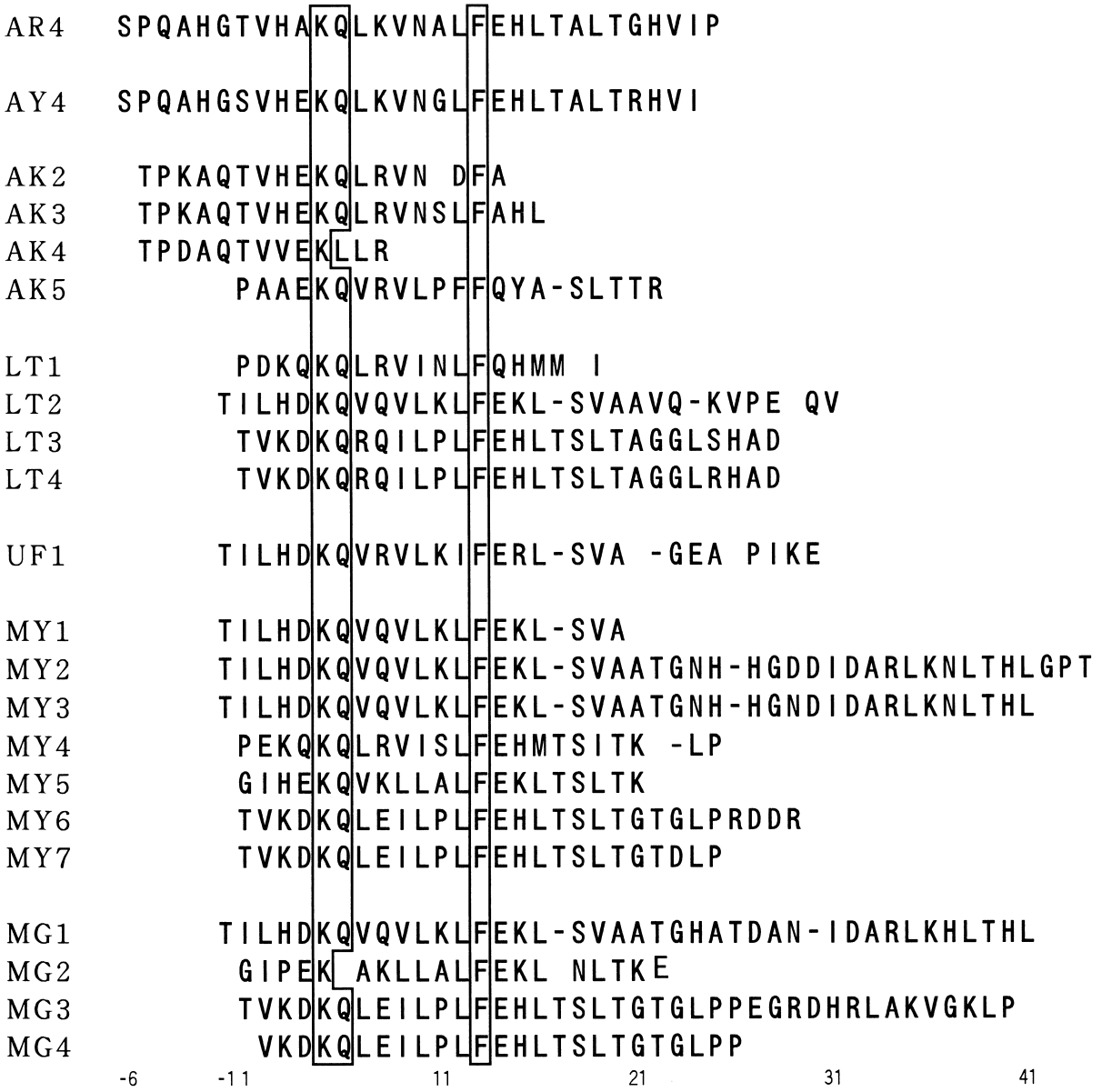

Fig. 2. N-terminal amino acid sequences of hemocyanin monomer subunits from *A. roretzi* (AR4), *A. yesoensis* (AY4), *A. karschi* (AK2–5), *L. typica* (LT1–4), *U. fragaria* (UF1), *M. yaginumai* (MY1–7) and *M. gigas* (MG1–4). The alignment was made by hand. Amino acid residues in blank position are ambiguous. Dashes represent gaps introduced under the necessity of comparing all N-terminal sequences. The conservative amino acid residues in positions 5, 6 and 13 are boxed.

viduals of each species including the polymorphic individuals (results not shown). In the present study, we could detect a total of twenty-nine hemocyanin monomer bands in Fig. 1 and analyzed the N-terminals of these monomer subunits. The N-terminal amino acid sequences of twenty-two monomer subunits were determined, because the subunits AR1–3, AY1–3 and AK1 could not be sequenced for the N-terminals, suggesting blocked amino terminals, which were reported in hemocyanin subunits of a scorpion (Ali *et al*., 1995) and araneomorph spiders (Takasu and Sugita, 1997). Direct protein sequencing of the hemocyanin monomers presented the sequences for the first 13–43 amino acid residues. The sequences of hemocyanin monomers from the mygalomorph spiders were aligned in Fig. 2.

The dimer subunits were distinguished from monomer subunits based on the relation between gel concentration and protein mobility in acrylamide gel electrophoresis. Figure 3 shows the effect of gel concentrations on mobility of the subunit proteins in the *L. typica* hemocyanin. If two molecules have different net charges but the similar molecular size, such plots as in Fig. 3 yield parallel lines (Sugita and Sekiguchi, 1975). From the results in Fig. 3, it is apparent that hemocyanin subunits LT1–4 have a molecular size similar to that of bovine serum albumin (67KDa) and that LT-D1 has a molecular size nearly equal to the dimer size of bovine serum albumin. Like the *L. typica* hemocyanin, the hemocyanins of other mygalomorph spiders also have one or two hemocyanin dimers which do not dissociate into monomer subunits in Davis' buffer system where most of hemocyanin molecules dissociate into monomers (Sugita and Sekiguchi, 1975). The hemocyanins of *A. roretzi*, *A. yesoensis*, *A. karschi*, *L. typica*, *U. fragaria*, *M. yaginumai* and *M. gigas* showed two (AR-D1, D2), two (AY-D1, D2), one (AK-D1), one (LT-D1), one (UF-D1), two (MY-D1, D2) and two (MG-D1, D2) dimer bands, respectively (Fig. 1), and there was no polymorphism in these dimer subunits. In this study, the hemocyanin dimers of *A. roretzi*, *A. karschi*, *L. typica* and *M. yaginumai* were analyzed. The hemocyanin dimers except AR-D1 and D2 showed two bands in SDS-PAGE, suggesting that they were heterodimers. Direct protein sequencing of AR-D1 and D2 gave a sequence and no signal for another sequence, suggesting AR-D1 and D2 were homodimers. The SDS-PAGE banding patterns of the constituent monomers of the dimers are shown in Fig. 4 and their constituent monomers of lower molecular weight are denoted "a" and those of higher molecular weight are denoted "b". The N-terminals of these ten subunits in dimers were analyzed and determined for the first 15–32 amino acid residues (Fig. 5).

Like most chelicerate hemocyanin subunits, the most of the mygalomorph spider hemocyanin subunits possessed lysine, glutamine and phenylalanine residues in positions 5, 6 and 13, respectively. LT-D1b contained an arginine residue in position 5 and a proline residue in position 13. In position 6 of AK4 contained a leucine. Amino acids in position 6 of MG2 and position 13 of MY-D1a were ambiguous. On the N-terminals, subunits AR4 and AY4 were six residues longer, AK2–4 were five residues longer, AR-D1 and AR-D2 were three residues longer, AK-D1b, LT-D1b, MY-D1b and MY-D2b were two residues longer, LT2, UF1, MY1–3, MG1, AK-D1a and LT-D1a were a residue longer, and MG4 was a residue shorter

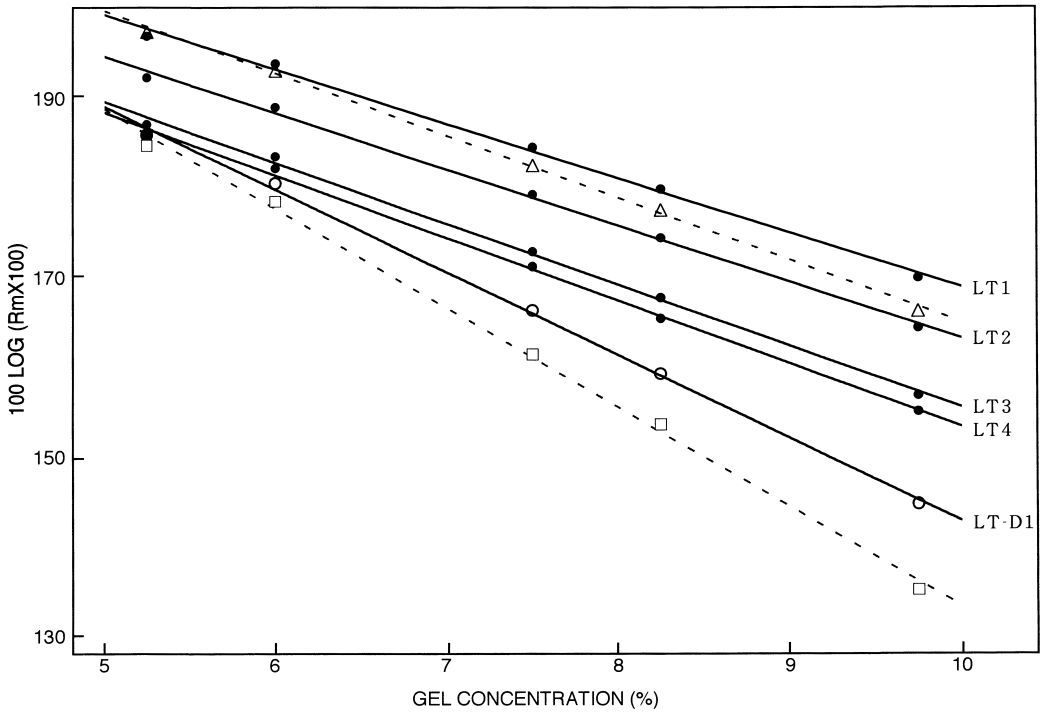

Fig. 3. The effect of various gel concentrations on the mobility of monomer and dimer subunits in the *L. typica* hemocyanin. ●, hemocyanin monomer; ○, hemocyanin dimer; △, bovine serum albumin monomer; □, bovine serum albumin dimer.

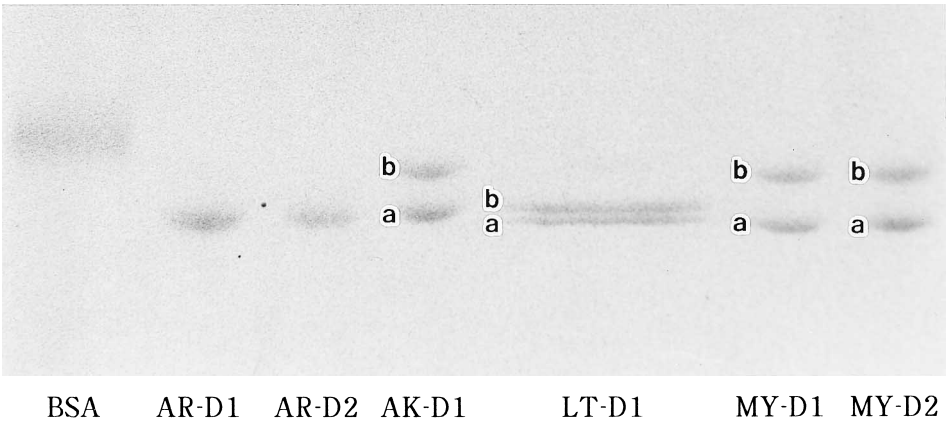

Fig. 4. SDS PAGE banding patterns of constituent monomers in hemocyanin dimer subunits in the slab gel. BSA is bovine serum albumin monomer (84KDa) in Bio-Rad's SDS-PAGE standards. AR-D1 and D2, AK-D1, LT-D1 and MY-D1 and D2 are hemocyanin dimer subunits reduced their disulfide bonds from *A. roretzi*, *A. karschi*, *L. typica* and *M. yaginumai*, respectively. The constituent monomers of lower molecular weight are denoted "a" and those of higher molecular weight are denoted "b".

```
AR-D1      MPSDPSEKQIRLLSWFEHLS
AR-D2      MPSDPSEKQIRLLSWFEHLS

AK-D1a       SPAAEKQVRVLPFFQYASLTTKDKF
AK-D1b     SPDASEKQARL S FYH

LT-D1a       PSTAEKQQRILPFFQFTSLSTKDKFGILVQRD
LT-D1b     PSDANERQARLL LP

MY-D1a       STAEKQQRILPF QFTTLSTRDRFGILVQRD
MY-D1b     PFDTNEKQARLSTLFEHS
MY-D2a       PTAEKQQRILPFFQFTTLSTKDKFGILVQRDP
MY-D2b     SFDTNEKQARLSTLFEHS
           1          11         21         31
```

Fig. 5. N-terminal amino acid sequences of constituent monomers in hemocyanin dimer subunits from *A. roretzi* (AR-D1 and D2), *A. karschi* (AK-D1a and D1b), *L. typica* (LT-D1a and D1b), and *M. yaginumai* (MY-D1a, D1b, D2a and D2b). The alignment was made by hand. Amino acid residues in blank position are ambiguous. The conservative amino acid residues in positions 6, 7 and 13 are boxed.

than the typical subunits of chelicerate hemocyanins.

DISCUSSION

The mygalomorph hemocyanin subunits are classified into eight groups, as shown in Table 1, based on characteristic amino acids that are present in the respective positions of all subunits in each group and absent in the corresponding positions of the other subunits (Theißen *et al.*, 1996).

The subunits of group A have an extension of six residues on the N-terminals as their characteristic amino acids and show the sequence similarity of 87%. The subunits of group B have an extension of five residues and show 77–94% similarities. These extensions prior to TVHEKQL as a consensus sequence between subunits of groups A and B in positions 1–7 must have been produced by the terminal addition.

The subunits of group C have a threonine in position –1 and show sequence similarities of 64–83% when MY2 is used for sequence comparison as a representative of *M. yaginumai*. The extension of a threonine residue is characteristic for the subunits of group C and there is a considerable possibility that an insertion of isoleucine or leucine in position 1 or 2 gave rise to the extension. Furthermore, these subunits possess one more characteristic residue of lysine in position 11. The N-terminal extension of a residue is also found in AK-D1a and LT-D1a. However, the amino acid in these extensions is a serine and a proline, suggesting that the extensions were due to independent events different from the insertion in group C.

Table 1. Comparison of N-terminal sequences of mygalomorph hemocyanin subunits within each group. Sequence data of *A. californicum* hemocyanin subunits ACa, b and e are from Voit and Feldmaier-Fuchs (1990), Sonner *et al.* (1990) and Schneider *et al.* (1983), respectively. Amino acid residues in blank position are ambiguous. Dashes represent gaps introduced under the necessity of comparing all N-terminal sequences. The characteristic amino acids of each group are shown white letters in black boxes. Positions with identical residues in most of the sequences of each group are boxed. In pairwise comparison, all comparable residues between two sequences are counted.

Group	Subunit	N-terminal sequence	% similarity
		-6 1 11 21	AR4
A	AR4	SPQAHGTVHAKQLKVNALFEHLTALTGHVIP	
	AY4	SPQAHGSVHEKQLKVNGLFEHLTALTRHVI	87
		-5 1 11 21	AK2 AK3
	AK2	TPKAQTVHEKQLRVN DFA	
B	AK3	TPKAQTVHEKQLRVNSLFAHL	94
	AK4	TPDAQTVVEKLLR	77 77
		1 11 21 31 41	LT2 UF1 MY1 MY2 MY3 MG1
	LT2	TILHDKQVQVLKLFEKLSVAAVQ-KVPE QV	
	UF1	TILHDKQVRVLKIFERLSVA -GEA PIKE	69
C	MY1	TILHDKQVQVLKLFEKLSVA	100 85
	MY2	TILHDKQVQVLKLFEKLSVAATGNH-HGDDIDARLKNLTHLGPT	72 64 100
	MY3	TILHDKQVQVLKLFEKLSVAATGNH-HGNDIDARLKNLTHL	72 64 100 98
	MG1	TILHDKQVQVLKLFEKLSVAATGHATDAN-IDARLKHLTHL	72 68 100 83 83
	ACa	TILHDKQVQALKLFEKLSVAATGEPVPADQIDERLRNITTLGPN	77 68 95 73 73 71
		1 11 21	LT1 MY4
	LT1	PDKQKQLRVINLFQHMM I	
D	MY4	PEKQKQLRVISLFEHMTSITK LP	78
	ACe	PDKQKQLRVISLFEHMTSINTPLP	83 87
		1 11 21 31	LT3 LT4 MY6 MY7 MG3
	LT3	TVKDKQRQILPLFEHLTSLTAGGLSHAD	
	LT4	TVKDKQRQILPLFEHLTSLTAGGLRHAD	96
E	MY6	TVKDKQLEILPLFEHLTSLTGTGLPRDDR	75 75
	MY7	TVKDKQLEILPLFEHLTSLTGTDLP	76 76 96
	MG3	TVKDKQLEILPLFEHLTSLTGTGLPPEGRDHRLAKVGKLP	71 71 90 96
	MG4	-VKDKQLEILPLFEHLTSLTGTGLPP	73 73 92 92 96
		1 11 21	MY5
F	MY5	GIHEKQVKLLALFEKLTSLTK	
	MG2	GIPEK AKLLALFEKL NLTKE	84
		1 11 21 31	AK5 AK-D1a LT-D1a MY-D1a MY-D2a
	AK5	-PAAEKQVRVLPFFQYASLTTR	
	AK-D1a	SPAAEKQVRVLPFFQYASLTTKDKF	91
G	LT-D1a	PSTAEKQQRILPFFQFTSLSTKDKFGILVQRD	64 68
	MY-D1a	-STAEKQQRILPFLQFTTLSTRDRFGILVQRD	60 58 87
	MY-D2a	-PTAEKQQRILPFFQFTTLSTKDKFGILVQRDP	62 68 94 90
	ACb	PSTAEKQVRILPFFQFTSLSTK LAGLGVLGRG	68 63 74 63 66
		-3 1 11	AR-D1 AR-D2 AK-D1b LT D1b MY-D1b
	AR-D1	MPSDPSEKQIRLLSWFEHLS	
	AR-D2	MPSDPSEKQIRLLSWFEHLS	100
H	AK-D1b	-SPDASEKQARL S FYH	63 63
	LT-D1b	-PSDANERQARLL LP	53 53 58
	MY-D1b	-PFDTNEKQARLSTLFEHS	53 53 60 64
	MY-D2b	-SFDTNEKQARLSTLFEHS	47 47 67 57 94

The subunits of group D show sequence similarities of 78-87% and characteristically contain glutamine, isoleucine and methionine residues in position 4, 10 and 16, respectively. The subunits of group E show sequence similarities of 71–96%. Although the subunits of group E possess no characteristic amino acid residue, they are fall into a same group because of their overall sequence traits distinctive from the other subunits. The subunits of group F possess a glycine residue in position 1 and an isoleucine residue in position 2 and they are absent in the corresponding positions of the other subunits.

The groups G and H are composed of the constituent monomers in dimer subunits except AK5. The subunits of group G possess characteristic residues of alanine, phenylalanine and leucine in positions 3, 12 and 18, respectively. The members of group G are the subunits in heterodimers (Fig. 4) except AK5. A hemocyanin subunit ACb of the tarantula spider *A. californicum* is also known as one component of a heterodimer (Sonner *et al.*, 1990) and is included in the group G. The subunits of group H possess the characteristic aspartic acid residue in position 1. The group H is composed of the constituent monomers in heterodimers (AK-D1, LT-D1, MY-D1 and D2) and in homodimers (AR-D1 and D2).

The characteristic amino acids are good candidates for synapomorphies in the respective groups, because the evolutionary events caused such characteristic changes as addition, insertion and substitution happened independently in an ancestral subunit of each group of hemocyanin subunits. In other words, the characteristic amino acids of a subunit group were derived from a common ancestral subunit. Therefore, it is considered that similar subunits with common characteristic amino acids among species are orthologous subunits which were descended from a subunit in a common ancestral species to extant species during the evolution of mygalomorph spiders.

Figure 6 shows the distribution of subunits of every group in mygalomorph spiders. The subunits of group A are distributed only in the genus *Antrodiaetus* of the family Antrodiaetidae, and the subunits of group B exist in *Atypus karschi* of the family Atypidae (Fig. 6A). Introducing a gap in position –1 or –2 of the hemocyanin subunits of group B, the N-terminally additional sequence of the subunits of group B

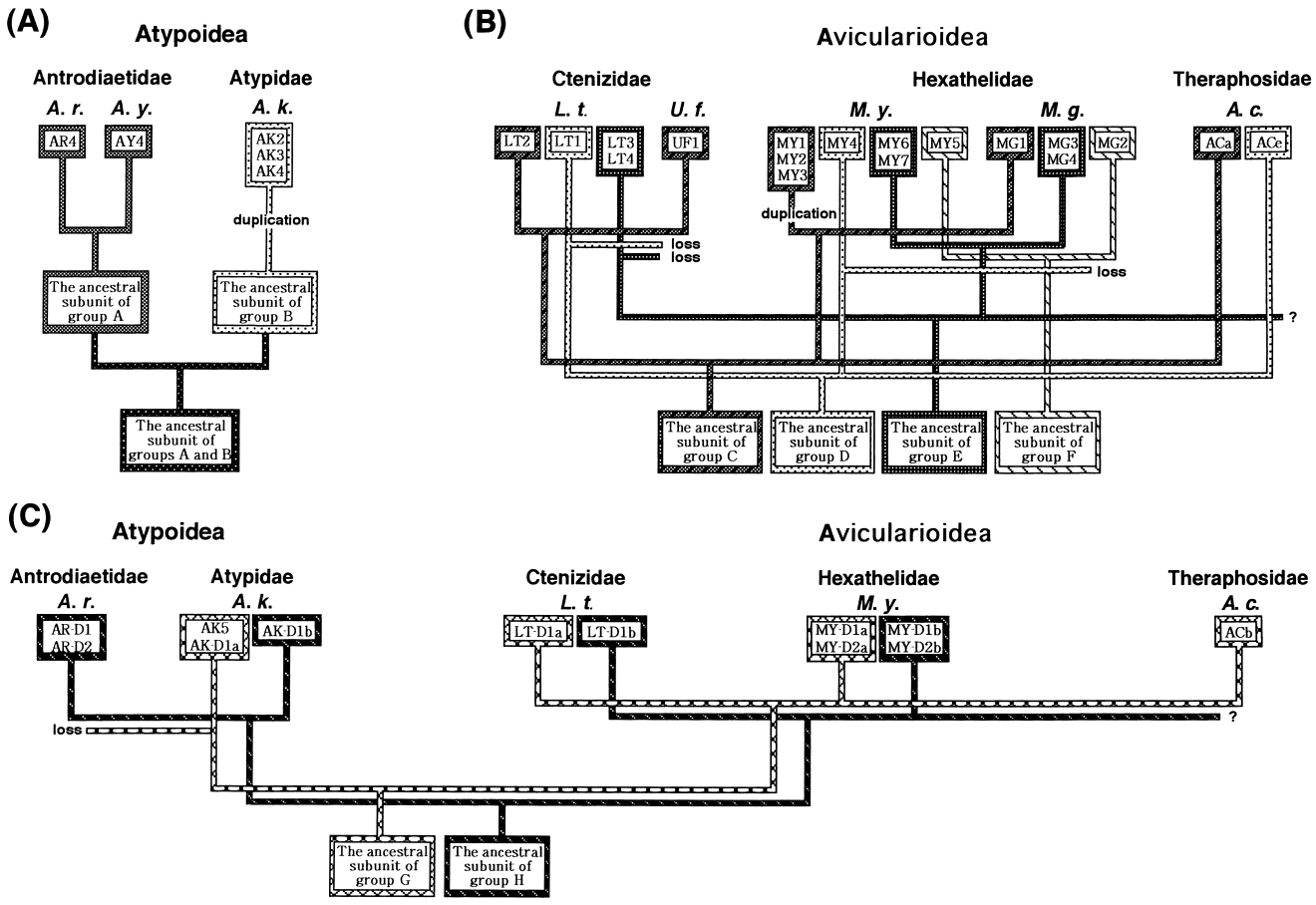

Fig. 6. The distribution of the hemocyanin subunits in mygalomorph spiders. The monomer subunits of groups A and B in Atypoidea (A), the monomer subunits of groups C, D, E and F in Avicularioidea (B) and constituent monomer subunits of groups G and H in Mygalomorphae (C) are shown separately. *A. r.*, *A. y.*, *A. k.*, *L. t.*, *U. f.*, *M. y.*, *M. g.* and *A. c.* denote *A. roretzi*, *A. yesoensis*, *A. karschi*, *L. typica*, *U. fragaria*, *M. yaginumai*, *M. gigas* and *A. californicum*, respectively. Cladistic classification of the mygalomorphs presented by Goloboff (1995) is used. The duplications and losses of the subunits were inferred from comparison of the subunit distribution patterns. ? means unknown sequences.

correspond well with that of the subunits of group A. Furthermore, the sequences following the N-terminal extensions of groups A and B are resemble each other, suggesting a consensus sequence of TVHEKQL, and the sequence similarity between the two groups is more than 64%. From these result, it is inferred that there is the close relationship between groups A and B, that is, the subunits of groups A and B have been originated from one subunit with an N-terminal extension which already existed in a common ancestor of the group Atypoidea. After divergence of the lineages of these families, an amino acid insertion or deletion occurred in position –1 or –2 of an ancestral hemocyanin subunit in the Antrodiaetidae or Atypidae lineage. Furthermore, it is thought from N-terminal sequence comparison within species that hemocyanin genes of group B were multiplied by duplication in the lineage of *A. karschi*, because *A. karschi* has the three similar subunits AK2–4 of group B.

The subunits of groups C and D are found widely in the families Ctenizidae, Hexathelidae and Theraphosidae of the group Avicularioidea, respectively (Fig. 6B), suggesting that the subunits of groups C and D originated respectively from one of the subunits of a common ancestor of these families. Furthermore, it is inferred that the subunits of group C were duplicated in the lineage of *M. yaginumai* and that the subunits of group D were lost in the lineages of *U. fragaria* and *M. gigas*, respectively. The subunits of group E are distributed in *L. typica* of the Ctenizidae and *Macrothele* of the Hexathelidae, and the subunits of group F exist only in the two species of the genus *Macrothele* (Fig. 6B). There was the loss of the subunit of group E in the lineage of *U. fragaria* (Fig. 6B). In this study, it is not clear whether the subunits of group E in *L. typica*, *M. yaginumai* and *M. gigas* were multiplied by the duplication in each lineage, because we cannot deny possibility of the existence of allelic products. However, there is a high possibility that the duplication of group E subunits occurred in the three lineages, because the hemocyanin of *L. typica* did not show any polymorphism in acrylamide gel electrophoresis and we used the hemocyanin samples of *M. yaginumai* and *M. gigas* which showed the least subunit bands in acrylamide gel electrophoresis.

The subunits of group G are found in Atypidae, Ctenizidae, Hexathelidae and Theraphosidae, and the subunits of group

H are found in Antrodiaetidae, Atypidae, Ctenizidae and Hexathelidae (Fig. 6C). Although the subunits of groups A–F are restricted to one group of either Atypoidea or Avicularioidea, the subunits of groups G and H are widely distributed in both groups of Atypoidea and Avicularioidea. Furthermore, it is probable that the constituent monomers of hemocyanin dimers were duplicated in *A. roretzi* and *M. yaginumai* lineage, respectively. However, although there was no polymorphism in the dimer subunits, we can not deny possibility of existence of allelic products. And the subunit of group G were not found in *A. roretzi*, inferring that the subunit of group G was lost in the *A. roretzi* lineage.

Reese and Mangum (1994) revealed that there was no relationship between intrinsic respiratory properties and subunit composition, and Lamy *et al*. (1983) showed that a hemocyanin hexamer could be made from homogeneous monomer subunits. Because some monomer subunits can self-aggregate into hexamers, the duplication and the loss of particular monomer subunits may do no significant damage to survival of these animals. Therefore, it is thought that the duplication and the loss of the hemocyanin subunits often occurred in the evolutionary history of mygalomorph spiders and have brought about the present state of distribution of hemocyanin subunits.

Markl (1980) found the disulfide-linked dimer subunits in spider hemocyanins and showed that the dimer subunits play an essential role in bonding one hexamer to another, suggesting that the dimer subunits are necessary to fix up quaternary structures of hemocyanins. Furthermore, Decker and Rimke (1998) found the *A. californicum* hemocyanin shows phenoloxidase activity and the function is limited to two subunits b and c which are components of a heterodimer. The heterodimer subunits of monomer components from both groups G and H have been conserved in all mygalomorph spiders examined except *A. roretzi* of which hemocyanin dimers are homodimers of subunits of group H. This means that the loss of dimer subunits was not allowed under functional restraint in evolutionary history of the mygalomorphs. However, the duplications of dimer subunits was allowed in the lineage of *M. yaginumai*, because it has done no harm to the spiders. Sonner *et al*. (1990) said that because of its key position within the oligomer, the study of the primary structure of subunits in dimers was of specific interest. It is necessary to analyze the complete amino acid sequences of subunits in dimers between species to clarify the molecular evolution of dimer subunits in detail. Our data will provide useful clues for future research of the evolutionary studies of mygalomorph hemocyanin subunits.

Although the duplication and deletion of hemocyanin subunits give some confusion in evolutionary studies of hemocyanin molecules, we clearly showed that it is possible to find hemocyanin subunits with similar traits among a numerous number of subunits based on the N-terminal sequence comparison. Furthermore, we can support the Goloboff's classification of mygalomorph spiders from the distribution patterns of the hemocyanin subunits, that is, the restricted distribution of the monomer subunits to one family (subunits of groups A or B) and to one group of Atypoidea (subunits of group A+B) or Avicularioidea (subunits of groups C–F), and the wide distribution of the dimer subunits to mygalomorph spiders, though Goloboff (1993) said that his reanalysis of mygalomorph spider families were considered too preliminary to warrant nomenclatural changes.

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REFERENCES

Ali SA, Zaidi ZH, Abbasi A (1995) Oxgen transport proteins: I. Structure and organization of hemocyanin from scorpion (*Buthus sindicus*). Comp Biochem Physiol 112A: 225–232

Beintema JJ, Stam WT, Hazes B, Smidt MP (1994) Evolution of arthropod hemocyanins and insect storage proteins (hexamerins). Mol Biol Evol 11: 493–503

Burmester T, Scheller K (1996) Common origin of arthropod tyrosinase, arthropod hemocyanin, insect hexamerin, and dipteran arylphorin receptor. J Mol Evol 42: 713–728

Davis BJ (1964) Disc electrophoresis-II. Method and application to human serum proteins. Ann N Y Acad Sci 121: 404–427

Decker H, Rimke T (1998) Tarantula hemocyanin shows phenoloxidase activity. J Biol Chem 273: 25889–25892

Goloboff PA (1993) A reanalysis of mygalomorph spider families (Araneae). Am Mus Novitates 3056: 1–32

Horn EC, Kerr MS (1969) The hemolymph proteins of the blue crab, *Callinectes sapidus*-I. Hemocyanins and certain other major protein constituents. Comp Biochem Physiol 29: 493–508

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Lamy J, Lamy J, Sizaret P-Y, Billiald P, Jollès P, Jollès J, Feldmann RJ, Bonaventura J (1983) Quaternary structure of *Limulus polyphemus* hemocyanin. Biochemistry 22: 5573–5583

Markl J (1980) Hemocyanins in spiders, XI. The quaternary structure of *Cupiennius* hemocyanin. J Comp Physiol 140: 199–207

Markl J, Decker H, Stöcker W, Savel A, Linzen B, Schutter WG, Van Bruggen EFJ (1981) On the role of dimeric subunits in the quaternary structure of arthropod hemocyanins. Hoppe-Seyler's Z Physiol Chem 362: 185–188

Raven RJ (1985) The spider infraorder Mygalomorphae (Araneae): Cladistics and systematics. Bull Am Mus Nat Hist 182: 1-180

Reese JE, Mangum CP (1994) Subunit composition and O_2 binding of the crustacean hemocyanins: Interspecific relationships. Biol Bull 187: 385–397

Schartau W, Eyerle F, Reisinger P, Geisert H, Storz H, Linzen B (1983) Hemocyanins in spiders, XIX. Complete amino-acid sequence of subunit *d* from *Eurypelma californicum* hemocyanin, and comparison to chain *e*. Hoppe-Seyler's Z Physiol Chem 364: 1383–1409

Schneider H-J, Drexel R, Feldmaier G, Linzen B, Lottspeich F, Henschen A (1983) Hemocyanins in spiders, XVIII. Complete amino-acid sequence of subunit *e* from *Eurypelma californicum* hemocyanin. Hoppe-Seyler's Z Physiol Chem 364: 1357–1381

Sonner P, Voit R, Schartau W (1990) Primary structure of subunit *b* of *Eurypelma californicum* hemocyanin. In: Préaux G, Lontie R (eds) Invertebrate Dioxygen Carriers. Leuven University Press, Leuven, pp 77–80

Sugita H, Sekiguchi K (1975) Heterogeneity of the minimum functional unit of hemocyanins from the spider (*Argiope bruennichii*),

the scorpion (*Heterometrus* sp.), and the horseshoe crab (*Tachypleus tridentatus*). J Biochem 78: 713–718

Sugita H, Murayama H (1998) N-terminal amino acid sequence comparison of Asian horseshoe crab hemocyanins: Immunologically identical hemocyanin subunits are orthologous in Asian horseshoe crabs. Zool Sci 15: 295–299

Sugita H, Kuwada T, Okamura K, Makioka T (1999) Identification of orthologous hemocyanin subunits in scorpions: The first step to produce a phylogenetic tree using amino acid sequence data. Species Diversity, 4: 35–42

Takasu Y, Sugita H (1997) N-terminal amino acid sequence comparison of hemocyanin subunits from the spiders, *Argiope amoena*, *Argiope bruennichii*, *Araneus ventricosus*, *Nephila clavata* and *Dolomedes sulfureus*. Acta Arachnol 46: 115–120

Theißen G, Kim JT, Saedler H (1996) Classification and phylogeny of the MADS-box multigene family suggest defined roles of MADS-box gene subfamilies in the morphological evolution of eukaryotes. J Mol Evol 43: 484–516

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76: 4350–4354

Van Bruggen EFJ, Bijlholt MMC, Schutter WG, Wichertjes T, Bonaventura J, Bonaventura C, Lamy J, Lamy J, Leclerc M, Schneider H-J, Markl J, Linzen B (1980) The role of structurally diverse subunits in the assembly of three cheliceratan hemocyanins. FEBS Litters 116: 207–210

Van Holde KE, Miller KI (1995) Hemocyanins. Adv Prot Chem 47: 1–81

Voit R, Feldmaier-Fuchs G (1990) Arthropod hemocyanins. Molecular cloning and sequencing of cDNAs encoding the tarantula hemocyanin subunits a and e. J Biol Chem 265: 19447–19452

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