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Hemocyanin Subunits of a Whipscorpion, *Typopeltis crucifer*, and a Primitive Spider, *Heptathela kimurai*: Orthologous Hemocyanin Subunits in Arachnids

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ABSTRACT—The presence of monomer and dimer subunits was revealed, by means of native polyacrylamide gel electrophoresis (PAGE), in examined arachnid hemocyanins. We determined the N-terminal amino acid sequences of nine monomer subunits prepared from hemolymph of a whipscorpion, *Typopeltis crucifer*, and a primitive spider, *Heptathela kimurai*, and two constituent monomers of a dimer subunit from the whipscorpion. Based on a comparison of the sequences, we confirmed that the orthologous hemocyanin subunits are shared between the whipscorpion and the scorpion, *Liocheles australasiae*, between the primitive spider and the scorpion, and among the whipscorpion, the scorpion, and mygalomorph spiders. This study is the first to demonstrate the presence of orthologus hemocyanin subunits in different orders. Furthermore, it is evident that one of the constituent monomers of the hemocyanin dimer from the whipscorpion is orthologous to the constituent monomers (the group G subunits) of the hemocyanin dimers in mygalomorph spiders and to the subunit LA8 (a constituent monomer of a dimer subunit) of the scorpion, suggesting that these constituent monomers of arachnid hemocyanin dimers originated from a common ancestral gene which existed in a common ancestor of these arachnids.

INTRODUCTION

Hemocyanins are the blue respiratory pigments found in the hemolymph of many arachnids including scorpions, whipscorpions, and spiders, and are organized as hexamers of monomer subunit chains or as multihexamers of these hexamers. The monomer subunits associate noncovalently in most cases, although a few hemocyanins contain one or more disulfide-linked dimers (Van Holde and Miller, 1995). The heterogeneity of the monomer subunits of spider and scorpion hemocyanins has been demonstrated by means of polyacrylamide gel electrophoresis (Sugita and Sekiguchi, 1975; Takasu and Sugita, 1997; Sugita *et al*., 1999; Kuwada and Sugita, 2000), 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).

The complete amino acid sequences of the hemocyanin subunits have been determined for several arachnids (Schartau *et al*., 1983; Schneider *et al*., 1983; Voit and Feldmaier-Fuchs, 1990; Buzy *et al*., 1995) and horseshoe crabs (Takagi and Nemoto, 1983; Nakashima *et al*., 1986). The identity percentages between these sequences range from 53 to 65 (Linzen *et al*., 1985; Voit and Feldmaier-Fuchs, 1990; Beintema *et al*., 1994; Buzy *et al*., 1995). Using the complete amino acid sequence data, phylogeny inference programs produced branching patterns for the hemocyanin subunits from a tarantula spider, *Aphonopelma* (as *Eurypelma*) *californicum*, and from horseshoe crabs, *Limulus polyphemus* and *Tachypleus tridentatus* (Beintema *et al*., 1994; Burmester and Scheller, 1996). However, the patterns could not reveal any evolutionary relation between animals having these subunits, while they showed evolutionary trees of hemocyanin monomer subunits.

On the other hand, N-terminal sequence analysis is an effective method to initiate research on the evolution of hemocyanin subunits. From comparison of the N-terminal amino acid sequences of hemocyanin subunits, the orthologous subunits which are available for determining animals' evolutionary trees have been found in araneomorph spiders (Takasu and Sugita, 1997), horseshoe crabs (Sugita and Murayama, 1998), scorpions (Sugita *et al*., 1999), and mygalomorph spiders (Kuwada and Sugita, 2000), respectively. This data provide the basis for discussion of the origins and duplications of the hemocyanin subunits during the evolution of animals having these subunits. However, the orthologous subunit shared among the arachnid hemocya-

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nins examined could not be detected, suggesting the necessity of analyzing hemocyanin subunits from the sister taxa of these animals.

In this paper, the hemocyanin subunits from a whipscorpion, *Typopeltis crucifer* Pocock (Thelyphonidae), and a primitive spider, *Heptathela kimurai* (Kishida) (Liphistiidae), are reported. We analyzed the N-terminal amino acid sequences of nine hemocyanin monomer subunits prepared from the two animals and two constituent monomers of the dimer subunit from the whipscorpion hemocyanin, and compared these sequences with known sequences of scorpion and spider hemocyanin subunits in order to find the orthologous subunits in arachnids.

MATERIALS AND METHODS

The whipscorpion, *T. crucifer*, was collected in Iriomote-jima Island, Okinawa Prefecture. The primitive spider, *H. kimurai*, was collected from Kagoshima City in Kagoshima Prefecture.

The legs of an animal were cut by a razor and the hemolymph bled from sections of the legs was sucked into a microsyringe. After removing sediments by centrifugation at 13,000 rpm for 5 min, the supernatant was maintained 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 described by Davis (1964), and 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). 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 were stained with 0.08% rubeanic acid in 41.7% methanol and 16.7% acetic acid according to the method described by Horn and Kerr (1969).

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 a sufficient amount of subunit protein for sequence analysis could not be 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 small amount of SDS-sample buffer containing 25 mM Tris, 0.1% SDS, and 0.1% 2-mercaptoethanol (pH 6.8). After being dialyzed 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 described by 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.

To collect the constituent monomers of the hemocyanin dimer, the dimer band in the gel was cut out, homogenized, and dialyzed against the SDS-sample buffer. The SDS sample was re-electrophoresed into the SDS slab gel in order to separate the constituent monomer peptides of the dimer. 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 and soaked in the transfer buffer. The transfer buffer contained 25 mM Tris, 192 mM 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 sequence analysis was performed using an Applied Biosystems model 447A or Procise 492 gas phase sequencer.

RESULTS

Fig. 1 shows the native PAGE banding patterns of a whipscorpion, *T. crucifer*, hemocyanin in various acrylamide concentrations ranging from 5.25 to 9.75%, while the relation between the relative mobilities of the protein bands and acrylamide concentration is shown in Fig. 2. If molecules have different net charges but a similar molecular size, such plots as those shown in Fig. 2 yield parallel lines (Sugita and Sekiguchi, 1975). From the results seen in Fig. 2, it is apparent that hemocyanin subunits TC1-TC6 have a molecular size similar to that of bovine serum albumin (67kDa), and that TCd1 has a molecular size definitely different from subunits TC1-TC6 and nearly equal to the dimer size of bovine serum albumin. The hemocyanin dimer TCd1 of *T. crucifer* separated into two bands in SDS PAGE, suggesting that the dimer was a heterodimer. The SDS PAGE banding pattern of the constituent monomers of the dimer is shown in Fig. 3, with the constituent monomer of lower molecular weight denoted as

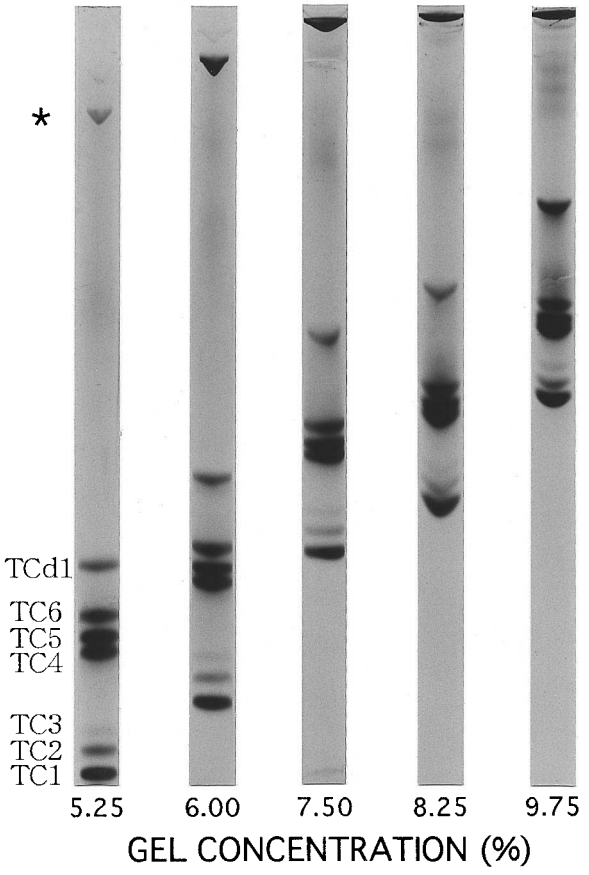

Fig. 1. Native PAGE banding patterns of *T. crucifer* hemocyanin subunits in various acrylamide gel concentrations. The hemocyanin monomer bands (TC1-TC6) were numbered from the bottom to the top of the gels, and the hemocyanin dimer band was named TCd1. The protein band with an asterisk is a hemocyanin oligomer band.

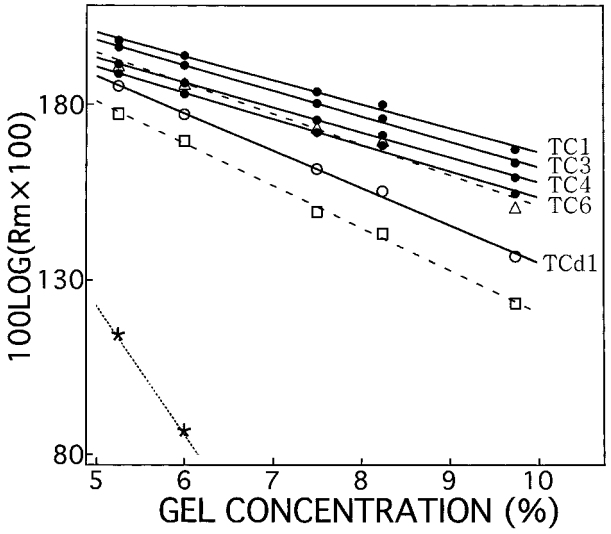

Fig. 2. The effect of various gel concentrations on the mobility of hemocyanin monomer and dimer subunits in the *T. crucifer* hemocyanin. The subunits of TC2 and TC5 were separated between TC1 and TC3 and between TC4 and TC6, respectively, and their slopes were almost equal to those of TC1, TC3, TC4, and TC6. ●, hemocyanin monomer; ○, hemocyanin dimer; △, bovine serum albumin monomer; □, bovine serum albumin dimer; ⋆, hemocyanin oligomer.

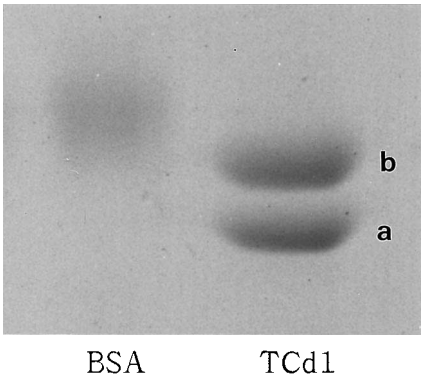

Fig. 3. SDS PAGE banding pattern of constituent monomers from *T. crucifer* hemocyanin dimer TCd1. BSA is the bovine serum albumin monomer (84KDa) in Bio-Rad's SDS-PAGE standards. The constituent monomer of lower molecular weight was denoted as "a" and that of higher molecular weight was denoted as "b".

"a" and that of higher molecular weight denoted as "b". The proteins in the band with an asterisk (Fig. 1) have much larger molecular sizes than the hemocyanin dimer. Because the protein band was stained by rubeanic acid (data not shown), the proteins in the band were determined as homogeneous hemocyanin oligomers.

The native PAGE banding patterns of a primitive spider, *H. kimurai*, and the relation between the relative mobilities of the protein bands and acrylamide concentration are shown in Figs. 4 and 5, respectively. From these results, it is evident that the hemocyanin of *H. kimurai* has four monomer subunits (HK1-HK4) and no dimer subunit. Three protein bands indicated by an asterisk in Fig. 4 give higher slopes than the bovine serum albumin dimer band in Fig. 5, respectively. The

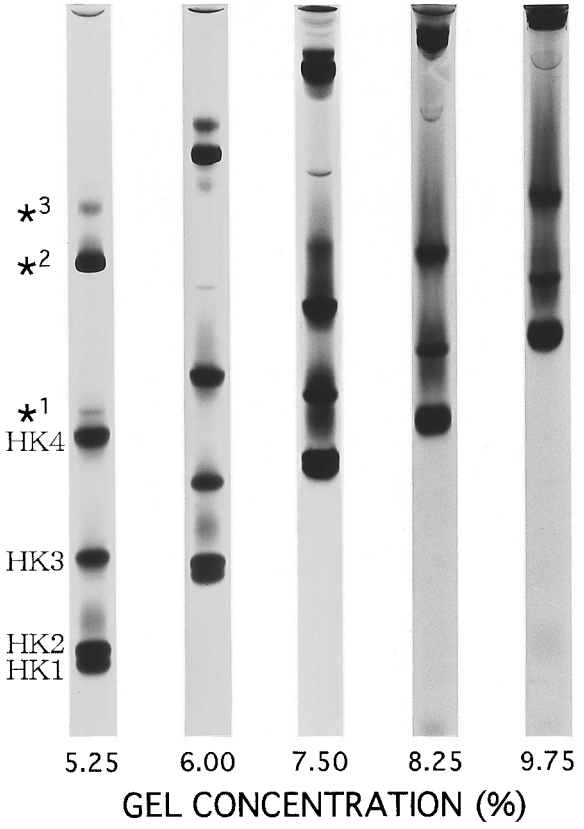

Fig. 4. Native PAGE banding patterns of *H. kimurai* hemocyanin subunits in various acrylamide gel concentrations. The hemocyanin monomer bands (HK1-HK4) were numbered from the bottom to the top of the gels. The protein band indicated by the asterisk 2 ($\star^2$) is a hemocyanin oligomer band. The proteins in two bands indicated by the asterisk 1 ($\star^1$) and the asterisk 3 ($\star^3$) are not hemocyanin, because they do not contain copper atoms.

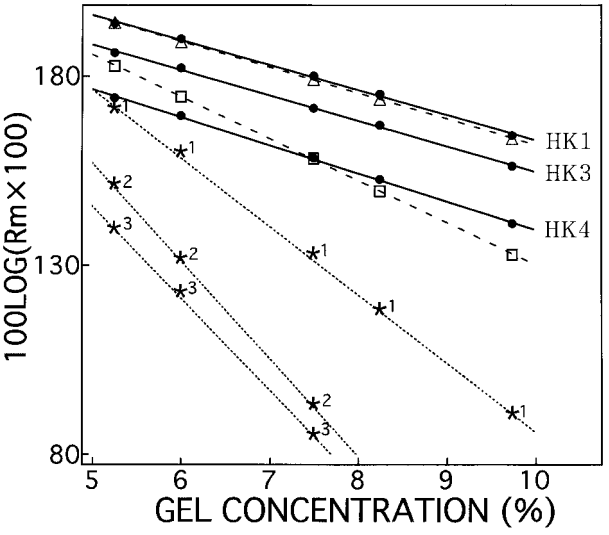

Fig. 5. The effect of various gel concentrations on the mobility of hemocyanin monomer subunits in *H. kimurai* hemocyanin. The subunit HK2 was separated between HK1 and HK3, and the slope of HK2 was almost equal to those of HK1, HK3, and HK4. ●, hemocyanin monomer; △, bovine serum albumin monomer; □, bovine serum albumin dimer; $\star^2$, hemocyanin oligomer; $\star^1$ and $\star^3$, the proteins contained no copper atoms.

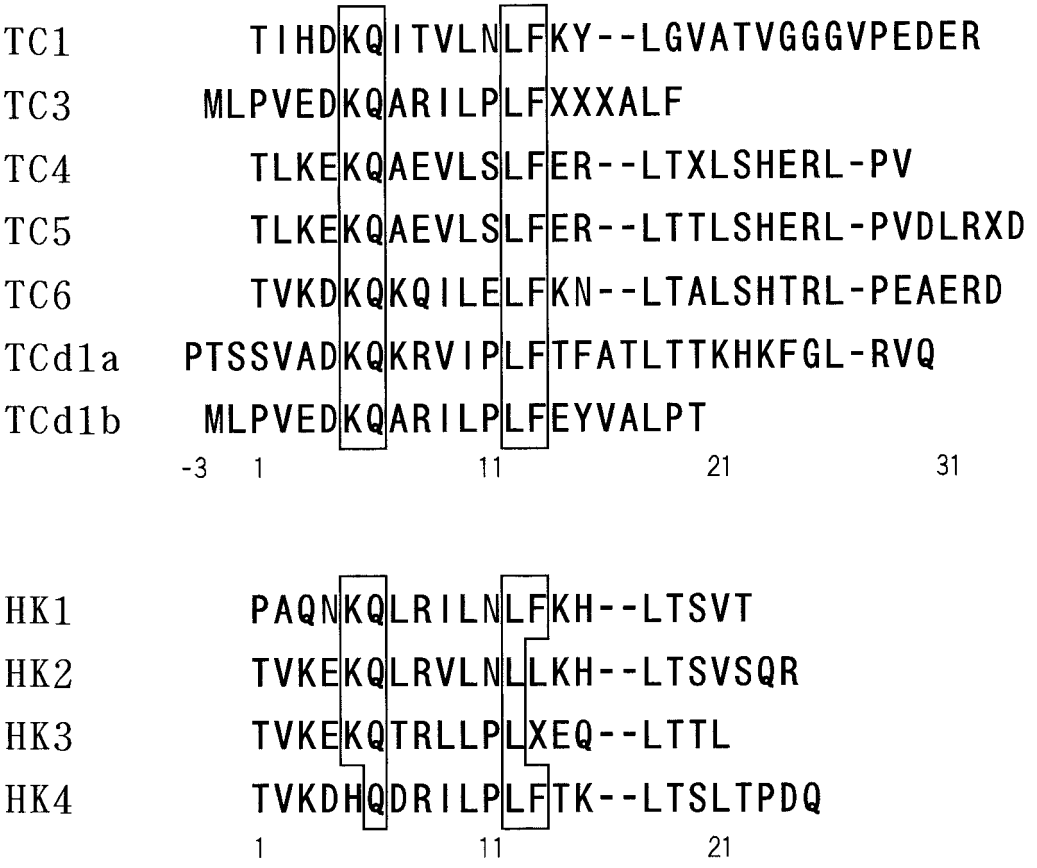

Fig. 6. N-terminal amino acid sequences of hemocyanin monomers from *T. crucifer* and *H. kimurai*, and constituent monomers (TCd1a and TCd1b) in the hemocyanin dimer subunit from *T. crucifer*. The alignment was made by hand. Amino acid residues indicated by X are ambiguous. Dashes represent gaps introduced under the necessity of comparing all N-terminal sequences. The conservative amino acid residues of most hemocyanin subunits from chelicerates (including arachnids and horseshoe crabs) are boxed in positions 5–6 and 12–13.

protein bands denoted by the asterisks 2 ($*^2$) contained copper atoms (data not shown). Therefore, only the proteins in the band with the asterisk 2 are hemocyanin oligomers. The slope given by the hemocyanin oligomer bands of *H. kimurai* is similar to that of *T. crucifer*.

The N-terminal amino acid sequences of the hemocyanin monomers and the constituent monomers of the dimer were analyzed and determined for the first 19–32 amino acid residues except TC2 (Fig. 6). Like most arachnid hemocyanin subunits, most of these hemocyanin subunits possessed lysine-glutamine and leucine-phenylalanine residues in positions 5–6 and 12–13, respectively. On the N-terminals, a subunit TCd1a was three residues longer and subunits TC3 and TCd1b were two residues longer than the typical subunits of arachnid hemocyanins, respectively.

DISCUSSION

In native PAGE, the whipscorpion, *T. crucifer*, hemocyanin also shows the heterogeneity of monomer subunits (Figs. 1–2) as well as other arachnid hemocyanins, and, in SDS PAGE, *T. crucifer* hemocyanin shows a heterodimer which consists of two different monomers as well as mygalomorph spider hemocyanins (Kuwada and Sugita, 2000).

Like the whipscorpion hemocyanin, the hemocyanin of the primitive spider, *H. kimurai*, shows an oligomer band, while *H. kimurai* hemocyanin does not show dimer band (Figs. 4–5). This suggests that *H. kimurai* hemocyanin dimer subunits associated noncovalently were dissociated and electrophoresed into the native acrylamide gel.

In the present study, the molecular size of the oligomers in *T. crucifer* and *H. kimurai* hemocyanins was not determined. However, the molecular weight of these oligomers may be 70–80 KDa × 24, because the major hemocyanins present at or near physiological pH are 24-mer subunits in scorpions, a whipscorpion, whipspiders, and in mygalomorph spiders (Markl *et al.*, 1979; Ellerton *et al.*, 1983).

The N-terminal sequence similarities of hemocyanin subunits are compared within species in Tables 1 and 2 and between species in Table 3. In the *T. crucifer* hemocyanin, the N-terminal amino acid sequence of subunit TC4 is identical to that of subunit TC5 as far as the N-terminals could be analyzed in this experiment, and the subunits TC3 and TCd1b have similar N-terminal sequences with a sequence similarity of 94% between them, although the other pairwise similarities are 30–60% (Table 1). The subunits of *H. kimurai* hemocyanin show the sequence similarities of 39–65% within species (Table 2). These values of 30–60% and 39–65% within spe-

Table 1. N-terminal similarity % scores among *T. crucifer* hemocyanin subunits (TC1, TC3-TC6, TCd1a, and TCd1b).

	TC1	TC3	TC4	TC5	TC6	TCd1a	TCd1b
TC1		47	36	38	45	30	44
TC3	7/15		47	47	60	50	94
TC4	9/25	7/15		100	60	36	47
TC5	11/29	7/15	25/25		55	37	50
TC6	13/29	9/15	15/25	16/29		37	50
TCd1a	8/27	9/18	9/25	10/27	10/27		45
TCd1b	8/18	17/18	8/17	9/18	9/18	10/22	

Values in the upper right half of the table are the similarity percentages between N-terminal amino acid sequences, and values in the lower left half of the table are the same residues/compared residues in a pairwise sequence comparison. In the pairwise comparison, gaps were inserted to maximize the similarity scores, but were not counted.

Table 2. N-terminal similarity % scores among *H. kimurai* hemocyanin subunits (HK1–HK4).

	HK1	HK2	HK3	HK4
HK1		65	39	50
HK2	13/20		61	45
HK3	7/18	11/18		61
HK4	10/20	10/22	11/18	

For explanations see Table 1.

Table 3. N-terminal similarity % scores between *T. crucifer* and *H. kimurai* hemocyanin subunits.

	HK1	HK2	HK3	HK4
TC1	45 (9/20)	41 (9/22)	33 (6/18)	35 (8/23)
TC5	35 (7/20)	50 (11/22)	67 (12/18)	39 (9/23)
TC6	45 (9/20)	50 (11/22)	56 (10/18)	52 (12/23)
TCd1a	35 (7/20)	36 (8/22)	50 (9/18)	43 (10/23)
TCd1b	50 (9/18)	39 (7/18)	59 (10/17)	56 (10/18)

Values in parentheses are the same residues/compared residues in a pairwise sequence comparison. TC3 and TC4 of *T. crucifer* hemocyanin are excluded from this comparison, because they show high sequence similarities with TCd1b and TC5, respectively. Other explanations are the same as those in Table 1.

cies are in the almost same range of the sequence similarities of 33–67% between *T. crucifer* and *H. kimurai* hemocyanin subunits (Table 3). Because *T. crucifer* hemocyanin subunits did not show any polymorphism in acrylamide gel electrophoresis, it is thought that duplications of subunit genes independently gave rise to the two pairs of TC4-TC5 and TC3-TCd1b in the *T. crucifer* lineage.

The orthologous hemocyanin subunits among many similar subunits can be detected by using 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 characteristic amino acids are good candidates for synapomorphies in the respective groups, because the evolutionary events that caused such characteristic changes happened independently in an ancestral subunit of each group of hemocyanin subunits. 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 the extant species during the evolution of animals. Based on characteristic amino acids and N-terminal sequence similarity scores, Kuwada and Sugita (2000) found the orthologous hemocyanin subunits among mygalomorph spiders and classified the mygalomorph hemocyanin subunits into eight groups. In the present study, we compared the N-terminal amino acid sequences of *T. crucifer* and *H. kimurai* hemocyanin subunits with those of scorpion and spider hemocyanin subunits in order to find the orthologous subunits in arachnids.

The subunit TC1 of *T. crucifer* and the subunit LA5 of the scorpion, *Liocheles australasiae*, have a characteristic sequence VATVGGGV at positions 18–25 and show sequence similarity of 63% (Fig. 7). The subunit HK3 of *H. kimurai* and the subunit LA1 of *L. australasiae* show sequence similarity of 78% and possess a characteristic glutamine residue at position 15 (Fig. 7). The subunit TCd1a of *T. crucifer* shows sequence similarities of 58% for a constituent monomer LA8 of *L. australasiae* hemocyanin dimer and 62% for a constituent monomer MYd2a of the hemocyanin dimer from mygalomorph spider, *Macrothele yaginumai*. The subunit MYd2a is a member of group G subunits which were one of the constituent monomers in mygalomorph spider's hemocyanin heterodimers (Kuwada and Sugita, 2000). These subunits, TCd1a, LA8, and MYd2a, characteristically contain alanine and leucine residues in positions 3 and 18, respectively (Fig. 7). When the N-terminal sequences of *T. crucifer* and *H. kimurai* hemocyanin monomer subunits are compared with those of the constituent monomers of *T. crucifer* hemocyanin dimer subunit, two gaps have to be introduced in positions 16 and 17 of the monomer subunits (Fig. 6), and this is true for mygalomorph spider's hemocyanin subunits (Kuwada and Sugita, 2000). Therefore, it is clear that an insertion of the two amino acid residues in these positions happened in a common ancestral subunit of TCd1a, LA8, and MYd2a, and caused

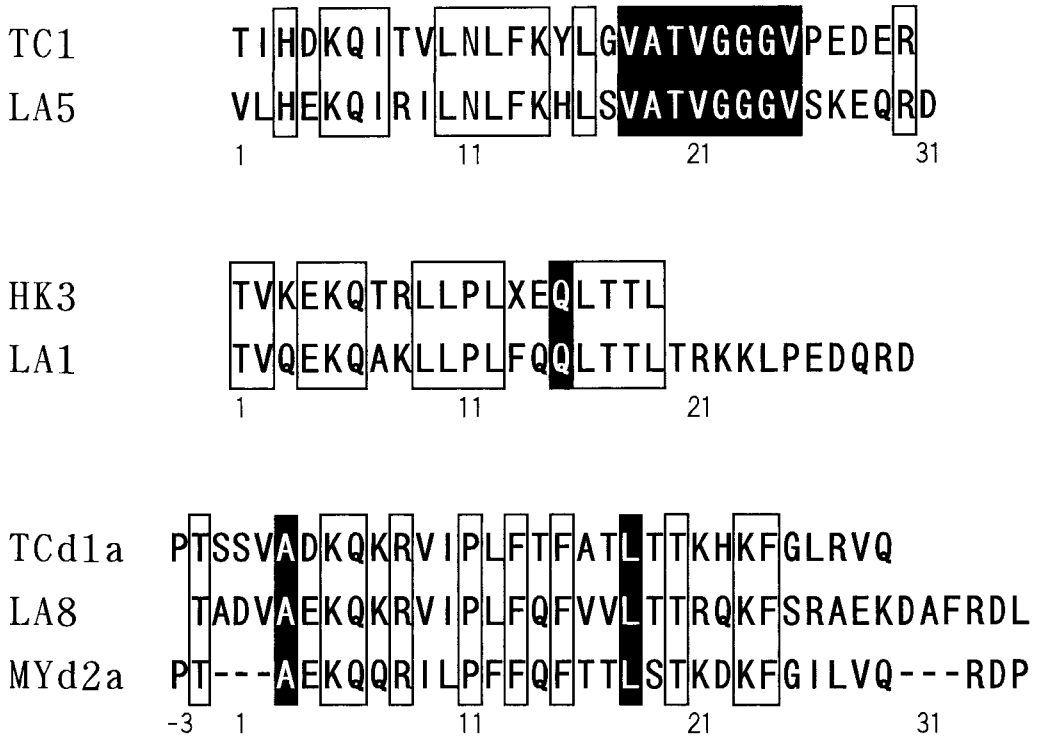

Fig. 7. Comparison of N-terminal sequences of orthologous hemocyanin subunits. Sequence data of LA1, LA5, and LA8 of *L. australasiae* are from Sugita *et al*. (1999), and MYd2a of *M. yaginumai* are from Kuwada and Sugita (2000). Amino acid residues indicated by X are ambiguous. Dashes represent gaps introduced under the necessity of comparing all N-terminal sequences. The characteristic amino acids of each group are shown by white letters in black boxes. Positions with identical residues in the sequences of each group are boxed.

the characteristic leucine residue in position 18 (Fig. 7). Furthermore, it could be considered that the insertion of two amino acid residues in the same positions occurred in the lineage of TCd1b, as well as in the lineages of TCd1a, LA8, and MYd2a, which possesses the characteristic leucine in position 18 (Fig. 6). So, this insertion must have shared the origin with the common insertion of TCd1a, LA8, and MYd2a. As mentioned above, the sequence traits of TC3 and TCd1b are similar to such a degree that we supposed that they were produced by gene duplication. If so, one of the duplications was allowed to lose its ability to form the dimer subunit and became the monomer subunit TC3.

In this study, we can find the orthologous subunits between the whipscorpion and the scorpion (TC1 and LA5), between the primitive spider and the scorpion (HK3 and LA1), and among the whipscorpion, the scorpion, and the mygalomorph spider (TCd1a, LA8, and MYd2a). This study is the first to detect the presence of orthologous hemocyanin subunits in arachnid orders.

It is interesting that the constituent monomers of the hemocyanin dimers, LA8, TCd1a, and MYd2a, are orthologous subunits which were derived from a common ancestral subunit. Markl (1980) found that the hemocyanin dimer plays an essential role in bonding one hexamer to another. That the constituent monomers of the hemocyanin dimer in arachnids originated from an ancestral subunits suggests that the function of the hemocyanin dimer was acquired in a common ancestral lineage of arachnids, and that hemocyanin dimers were conserved under some functional restraint in the evolutionary history of these animals. However, the hemocyanin dimer was not found in *H. kimurai* hemocyanin. It is necessary to analyze the complete amino acid sequences of hemocyanin subunits in order to clarify the molecular evolution of monomer and dimer subunits in detail.

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