Molecular Evolution of the Hemocyanin Subunits and Higher Classification in Mygalomorphae

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Contents

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. To investigate the evolution of the mygalomorph spiders based on amino acid sequences of hemocyanin subunits, the orthologous subunits derived from a common ancestral molecule must be identified and used for phylogenetic study. Based on the N-terminal sequence comparison of the monomer subunits and the constituent monomers of the hemocyanin dimers, the eight groups (groups A-H), each group of which consisted of the orthologous subunits, were found in the mygalomorph spiders. From the limited distribution patterns of the monomer subunits (groups A-F), it is evident that duplications and losses of the monomer subunits occurred frequently in the mygalomorph spiders. On the other hand, the constituent monomers of the hemocyanin dimers were distributed widely in the mygalomorph spiders, so that these subunits of groups G and H are available for phylogenetic analysis of the mygalomorph spiders as a whole. The phylogenetic analysis using amino acid sequences of the group G subunits supported the higher classification of the mygalomorph spiders proposed by Goloboff (1993) but did not the classification by Raven (1985). Although the duplication and deletion of the hemocyanin subunits give some confusion in evolutionary studies of hemocyanin molecules, the resulting distribution patterns of the hemocyanin monomers (group A-H) also supported the Goloboff's classification. The gene trees of the hemocyanin subunits showed the monophyly of the subunits of groups G and H and also showed the monophyly of chelicerate hemocyanin subunits including subunits of groups G and H. Thus it is inferred

that both of the constituent monomers (groups G and H) of the hemocyanin dimers were descended from a common ancestral gene and that the hemocyanin dimers were acquired independently in the two lineages of Chelicerata and Crustacea. Therefore, the constituent monomer of the hemocyanin dimers must be conserved in Chelicerata under functional restriction as a linker molecule between two hexamers of hemocyanins.

General Introduction

Hemocyanins are the blue respiratory pigments found in the hemolymph of many arthropods and are organized as hexamers of monomer subunit chains or as multiples 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 arthropod hemocyanins was demonstrated by means of polyacrylamide gel electrophoresis, but each of these subunits has a mass of about 75 kDa and carries one oxygen-binding site, suggesting that all are clearly derived from a common ancestral protein (Van Holde and Miller, 1995). In arthropods, it became clear that their hemocyanins contain one or more noncovalently linked or 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).

The complete amino acid sequences of hemocyanin monomers were determined in chelicerates (Schartau *et al.*, 1983; Schneider *et al.*, 1983; Linzen *et al.*, 1985; Nakashima *et al.*, 1986; Voit and Feldmaier-Fuchs, 1990; Buzy *et al.*, 1995) and crustaceans (Bak and Beintema, 1987; Jekel *et al.*, 1988; Neuteboom *et al.*, 1992; Sellos *et al.*, 1997). The sequences of these hemocyanin monomers show significant similarity to those of phenoloxidase (Aspán *et al.*, 1995; Fujimoto *et al.*, 1995; Hall *et al.*, 1995; Kawabata *et al.*, 1995), insect hexamerin (Sakurai *et al.*, 1988; Fujii *et al.*, 1989; Willott *et at.*, 1989; Jones *et al.*, 1990; Naumann and Scheller, 1991; Jones *et al.*, 1993; de Kort and Koopmanschap, 1994; Koopmanschap *et al.*, 1995; Jamroz *et al.*, 1996), dipteran arylphorin receptor (Maschat *et al.*, 1990; Chung *et al.*, 1995; Burmester and Scheller, 1996), cryptocyanin

(Terwilliger *et al.*, 1999) and pseudo-hemocyanin (Burmester, 1999). Phylogenetic analysis indicated that the hemocyanin subunits have evolutionary link with these proteins (Beintema *et al.*, 1994; Burmester and Scheller, 1996; Jamroz *et al.*, 1996; Burmester, 1999; Hughes, 1999; Terwilliger *et al.*, 1999). The branching patterns showed the relation between hemocyanins and the other proteins but did not have any information on the evolution of arthropod species having the hemocyanin subunits.

On the other hand, Sugita (1986, 1988) reported that each hemocyanin of three Asian horseshoe crabs is composed of six immunologically different subunits, each subunit of which is immunologically identical with the comparable subunits of two other species. Furthermore, the amino acid sequence analysis showed that immunologically identical subunits are orthologous subunits which were descended from a common ancestral sequences (Sugita and Shishikura, 1995; Sugita and Murayama, 1998). Therefore, we must choose orthologous hemocyanin subunits from many homologous subunits in order to analyze the divergence pattern of animals having these subunits, and then we can understand the molecular evolution of the hemocyanin subunits in detail by comparing all the subunits, that is, orthologous and paralogous sequences of the hemocyanin subunits.

In this study, I report the amino acid sequences of hemocyanin subunits from the mygalomorph spiders and discuss the higher classification of the mygalomorph spiders and the evolution of the hemocyanin subunits.

Part 1. Orthologous hemocyanin subunits in Mygalomorphae

1.1. Introduction

A mygalomorph spider, *Aphonopelma 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 monomer 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 the 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 monomer subunit b (Sonner *et al.*, 1990), which made up the heterodimer with the monomer 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; Jamroz *et al.*, 1996). However, the patterns could not show any evolutionary relation between animals having these subunits, while they showed evolutionary trees of hemocyanin monomer subunits.

On the other hand, from comparison of N-terminal amino acid sequences of the 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, they could discuss origins and duplications of the hemocyanin subunits

during the evolution of animals having these subunits. The N-terminal sequence analysis is an effective method to initiate research on the evolution of hemocyanin subunits.

In the present study, as the first step to study the evolution of hemocyanin subunits in the mygalomorph spiders, I 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 patterns of these subunits in the Mygalomorphae, I discuss the evolution of hemocyanin monomer and dimer subunits.

1.2. Materials and Methods

1.2.1. Materials

The antrodiaetids, *Antrodiaetus roretzi* (L. Koch, 1878) and *Antrodiaetus yesoensis* (Uyemura, 1942) were collected at Mt. Tsukuba, Ibaraki Prefecture and in Sapporo City, Hokkaido Prefecture, respectively. The atypid, *Atypus karschi* Dönitz, 1887 was collected in Tsukuba City, Ibaraki Prefecture. The ctenizids, *Latouchia typica* (Kishida, 1913) and *Ummidia fragaria* (Dönitz, 1887) were collected in Zushi City, Kanagawa Prefecture and at Mt. Tsukuba, Ibaraki Prefecture, respectively. The hexathelids, *Macrothele gigas* Shimojana & Haupt, 1998 and *Macrothele yagimumai* Shimojana & Haupt, 1998 were collected in Ishigaki-jima Island, Okinawa Prefecture and Iriomote-jima Island, Okinawa Prefecture, respectively.

1.2.2. Methods

1.2.2.1. Preparation of hemocyanin samples

The legs of a spider were cut by a razor 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 67 mM Tris, 13 mM 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.

1.2.2.2. Identification of hemocyanin subunits

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 the hemocyanin dimer subunits from the 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).

1.2.2.3. N-terminal amino acid sequences

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 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.

To collect the constituent monomers of the hemocyanin dimers, 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 and soaked in the transfer buffer. The transfer buffer contained 25 mM Tris, 192 mM glycine, 4% methanol, and 0.02% SDS (pH 8.3). Electrophoretic transfer was carried out for 7 h 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.

1.3. Results

1.3.1. Identification of hemocyanin subunits

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. Figure 1 shows the native PAGE banding patterns stained by CBB of hemocyanin samples from seven mygalomorph spiders. Protein bands with arrowheads contained hemocyanin molecules, because these protein bands were also stained by rubeanic acid (results not shown).

1.3.1.1. Hemocyanin subunits in *Antrodiaetus roretzi*

Figure 2 shows the native PAGE banding patterns of *A. roretzi* hemocyanin in various acrylamide concentrations ranging from 5.25 to 9.75%, and the relation between the relative mobility of the protein bands and acrylamide concentration is shown in Figure 3. If molecules have different net charges but a similar molecular size, such plots as those shown in Figure 3 yield parallel lines (Sugita and Sekiguchi, 1975). From the results seen in Figures 2 and 3, it is apparent that hemocyanin subunits AR1-AR4 have a molecular size similar to that of bovine serum albumin (67 kDa) and that ARd1 and ARd2 have a molecular size definitely different from subunits AR1-AR4 and nearly equal to the dimer size of bovine serum albumin. Therefore, *A. roretzi* hemocyanin consists of four monomers (AR1-4) and two dimers (ARd1 and ARd2). The proteins in the band with an asterisk (Fig. 2) have much larger molecular size than the hemocyanin dimer and contain copper, suggesting that they are hemocyanin oligomers. The native PAGE banding

patterns of the hemocyanin subunits of *A. roretzi* did not show any polymorphism.

1.3.1.2. Hemocyanin subunits in *Antrodiaetus yesoensis*

The native PAGE banding patterns of *A. yesoensis*, and the relation between the relative mobilities of the protein bands and acrylamide concentration are shown in Figures 4 and 5, respectively. From these results, it is evident that the hemocyanin of *A. yesoensis* has four monomer subunits (AY1-AY4) and two dimer subunits (AYd1 and AYd2). Because the proteins in the band with an asterisk (Fig. 4) have much larger molecular size than the hemocyanin dimer, the proteins in the band are hemocyanin oligomers. The native PAGE banding patterns of the hemocyanin subunits of *A. yesoensis* did not show any polymorphism.

1.3.1.3. Hemocyanin subunits in *Atypus karschi*

The native PAGE banding patterns of *A. karschi*, and the relation between the relative mobilities of the protein bands and acrylamide concentration are shown in Figures 6 and 7, respectively. From these results, it is evident that the hemocyanin of *A. karschi* has five monomer subunits (AK1-AK5) and one dimer subunit (AKd1). The proteins in the band with an asterisk (Fig. 6) have much larger molecular size than the hemocyanin dimer, showing proteins in the band are hemocyanin oligomers. The native PAGE banding patterns of the hemocyanin monomer subunits of *A. karschi* showed the polymorphism which was caused by allelic products. The banding pattern of the hemocyanin shown in Figure 6 was the most usual in *A. karschi*. Furthermore, the protein bands shown in

Figure 6 were found in all individuals including the polymorphic individuals (results not shown).

1.3.1.4. Hemocyanin subunits in *Latouchia typica*

The native PAGE banding patterns of *L. typica*, and the relation between the relative mobilities of the protein bands and acrylamide concentration are shown in Figures 8 and 9, respectively. From these results, it is evident that the hemocyanin of *L. typica* has four monomer subunits (LT1-LT4) and one dimer subunit (LTd1). The proteins in the band with an asterisk (Fig. 8) are hemocyanin oligomers. The native PAGE banding patterns of the hemocyanin subunits of *L. typica* did not show any polymorphism.

1.3.1.5. Hemocyanin subunits in *Ummidia fragaria*

The native PAGE banding patterns of *U. fragaria*, and the relation between the relative mobilities of the protein bands and acrylamide concentration are shown in Figures 10 and 11, respectively. From these results, it is evident that the hemocyanin of *U. fragaria* has one monomer subunit (UF1) and one dimer subunit (UFd1). The hemocyanin oligomer band is shown with an asterisk (Fig. 10). The native PAGE banding patterns of the hemocyanin subunits of *U. fragaria* did not show any polymorphism.

1.3.1.6. Hemocyanin subunits in *Macrothele gigas*

The native PAGE banding patterns of *M. gigas*, and the relation between the relative mobilities of the protein bands and acrylamide concentration are shown in Figures 12 and

13, respectively. From these results, it is evident that the hemocyanin of *M. gigas* has four monomer subunits (MG1-MG4) and two dimer subunits (MGd1 and MGd2). The protein band with an asterisk (Fig. 12) is hemocyanin oligomer band. The native PAGE banding patterns of the hemocyanin monomer subunits of *M. gigas* showed the polymorphism. The banding pattern of the hemocyanin shown in Figure 12 was the most usual in *M. gigas* and the hemocyanin bands in Figure 12 were found in all individuals including the polymorphic individuals (results not shown).

1.3.1.7. Hemocyanin subunits in *Macrothele yaginumai*

The native PAGE banding patterns of *M. yaginumai*, and the relation between the relative mobilities of the protein bands and acrylamide concentration are shown in Figures 14 and 15, respectively. From these results, it is evident that the hemocyanin of *M. yaginumai* has seven monomer subunits (MY1-MY7) and two dimer subunits (MYd1 and MYd2). The proteins in the band with an asterisk (Fig. 14) are hemocyanin oligomers. Although the polymorphism of the hemocyanin monomer subunits of *M. yaginumai* was found, the banding pattern of the hemocyanin shown in Figure 14 was the most usual in *M. yaginumai*. Furthermore, the protein bands shown in Figure 14 were found in all individuals including the polymorphic individuals (results not shown).

1.3.2. Analysis of hemocyanin dimers

In the present study, the hemocyanin dimers of *A. roretzi*, *A. karschi*, *L. typica* and *M. gigas* were analyzed as representatives of each family for further study. The hemocyanin

dimers except ARd1 and ARd2 showed two bands in SDS PAGE, suggesting that they were heterodimers. Direct protein sequencing of ARd1 and ARd2 gave a sequence and no signal for another sequence, suggesting ARd1 and ARd2 were homodimers. The SDS PAGE banding patterns of the constituent monomers of the dimers are shown in Figure 16 and their constituent monomers of lower molecular weight are denoted as "a" and those of higher molecular weight are denoted as "b".

1.3.3. N-terminal amino acid sequences of hemocyanin monomer subunits and constituent monomers of hemocyanin dimers

In the present study, I could detect a total of twenty-nine hemocyanin monomer subunits and ten constituent monomers of hemocyanin dimers and analyzed the N-terminals of these proteins. The N-terminal amino acid sequences of twenty-two monomer subunits were determined, because the subunits AR1-AR3 of *A. roretzi*, AY1-AY3 of *A. yesoensis* and AK1 of *A. karschi* 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 the hemocyanin monomers from the mygalomorph spiders were aligned in Figure 17. The N-terminals of ten constituent monomers of hemocyanin dimers were analyzed and determined for the first 15-32 amino acid residues (Fig. 18).

Like most chelicerate hemocyanin subunits, most of the mygalomorph spider

hemocyanin subunits possessed lysine, glutamine and phenylalanine residues in positions 5, 6 and 13, respectively. LTd1b contained an arginine residue in position 5 and a proline residue in position 13. In position 6 of AK4 contained a leucine. Amino acid in position 6 of MG2 was ambiguous. On the N-terminals, subunits AR4 and AY4 were six residues longer, AK2-AK4 were five residues longer, ARd1 and ARd2 were three residues longer, AKd1b, LTd1b, MGd1b and MGd2b were two residues longer, LT2, UF1, MG1, MY1-MY3, AKd1a, LTd1a, MGd1a and MGd2a were a residue longer, and MG4 was a residue shorter than the typical subunits of chelicerate hemocyanins.

1.4. Discussion

1.4.1. Identification of orthologous hemocyanin subunits

The mygalomorph hemocyanin subunits are classified into eight groups, as shown in Table 1, based on sequence similarities and 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 AKd1a, MGd1a, MGd2a, LTd1a and ACb. 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.

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 have no characteristic amino acid. However, because of their overall sequence similarities of 71-96%, they are fall into a same group. 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 of the 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. 16) 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. Although the subunits of group H 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 group H is composed of the constituent monomers of heterodimers (AKd1, LTd1, MGd1 and MGd2) and homodimers (ARd1 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. I think that each of groups A-H is

composed of the orthologous hemocyanin subunits available for making phylogenetic trees of mygalomorph spiders having these subunits, though the subunits of groups E and H have no characteristic amino acid.

1.4.2. Distribution of orthologous hemocyanin subunits

Figure 19 shows the distribution of subunits of every group in the 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. 19A). 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 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 results, 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 spiders in the two families. 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-AK4 of group B.

The subunits of groups C and D are found widely in the families Ctenizidae, Hexathelidae and Theraphosidae, respectively (Fig. 19B), suggesting that the subunits of groups C and D originated respectively from one of the subunits of a common ancestor of these three 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. 19B). There was the loss of the subunit of group E in the lineage of *U. fragaria* (Fig. 19B). In the present 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 I 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. 19C). Although the subunits of groups A and B are restricted to Antrodiaetidae and Atypidae and those of groups C-F are restricted to three other families, the subunits of groups G and H are widely distributed in the Mygalomorphae. Furthermore, it is probable that the constituent monomers of the

hemocyanin dimers were duplicated in *A. roretzi* and *M. gigas* lineage, respectively. However, although there was no polymorphism in the dimer subunits, I can not deny possibility of existence of allelic products. And the subunit of group G was not found in *A. roretzi*, suggesting that the subunit of group G was lost in the *A. roretzi* lineage.

From the present state of the distribution of the mygalomorph hemocyanin subunits, it is thought that the duplication and the loss of the hemocyanin subunits often occurred in the evolutionary history of the mygalomorph spiders (Fig. 19). These evolutionary events give some confusion for making evolutionary trees based on sequence data of hemocyanin subunits. Furthermore, the subunits of groups A-F, each group of which consists of orthologous subunits, were unavailable for phylogenetic analysis of the mygalomorph spiders as a whole because of their restricted distributions. However, the subunits of groups A and B and groups C and D are available for phylogenetic analysis between families Antrodiaetidae and Atypidae and among families Ctenizidae, Hexathelidae and Theraphosidae, respectively.

On the other hand, the constituent monomers of the hemocyanin dimers, that is subunits of groups G and H, were widely distributed in the mygalomorph spiders, so that the amino acid sequence comparison among of the subunits included in groups G and/or H are useful for making phylogenetic tree of the mygalomorph spiders as a whole.

Part 2. Higher classification of Mygalomorphae and the evolution of constituent monomers of hemocyanin dimers

2.1. Introduction

The mygalomorph spiders had been traditionally divided into atypoids including three families Atypidae, Antrodiaetidae and Mecicobotheriidae and non-atypoids including the rest of the mygalomorph spiders. The higher classification of the spider infraorder Mygalomorphae was cladistically analyzed by Raven (1985), which was the first to attempt an understanding of the relationships within the Mygalomorphae as a whole. Raven (1985) divided the mygalomorph spiders into two groups, Fornicephalae and Tuberculotae (Fig. 20A). Goloboff (1993) reanalyzed the relationships of mygalomorph spider families based on cladistic analysis by using computer programs. He suggested that neither Fornicephalae nor Tuberculotae were monophyletic groups, and resurrected the group Atypoidea, which was restricted to Antrodiaetidae and Atypidae, and the group Avicularioidea including the remaining families of the Mygalomorphae (Fig. 20B). Their analyses based on morphological characters revealed three monophyletic groups, Rastelloidina including Ctenizidae, Theraphosoidina including Theraphosidae and one more group named Atypoidina (Raven, 1985) or Atypoidea (Goloboff, 1993) including only two families Atypidae and Antrodiaetidae. However, these analyses did not reach the consensus of interpreting the relationships within the mygalomorphs as a whole.

The evolutionary relationships among arthropod hemocyanins and other homologous proteins such as hexamerins, phenoloxidase, and etc. were investigated (Beintema *et al.*,

1994; Burmester and Scheller, 1996; Jamroz *et al.*, 1996; Burmester, 1999; Hughes, 1999; Terwilliger *et al.*, 1999), and these study indicated that chelicerate hemocyanin monomer subunits constitute a monophyletic group. However, the divergence patterns of the animals having the hemocyanin monomer subunits showed strange figures, which were not consistent with the evolution of animal species. On the other hand, the hemocyanin dimers are known as "linker" molecules, which bond hexamers together (Markl, 1980; Van Bruggen *et al.*, 1980; Markl *et al.*, 1981). In *A. californicum* hemocyanin, the heterodimer bc showed phenoloxidase activity after limited proteolysis with trypsin and chymotrypsin, but other monomer subunits did not show the activity (Decker and Rimke, 1998). Therefore, it is expected that the constituent monomers of the hemocyanin dimers underwent distinct selection pressure from other hemocyanin monomer subunits. In fact, I gave evidences in the part one of this thesis that the constituent monomers of the hemocyanin dimers are divided into two groups G and H which show wider distribution in the Mygalomorphae than the other groups A-F, each group of which consisted of the monomer subunits. Furthermore, I showed that each of groups G and H consists of orthologous constituent monomers of the dimers. However, the evolutionary relationships between the two constituent monomers of heterodimers were not discussed.

In the present study, in order to investigate the evolutionary history of the mygalomorph spiders, the partial amino acid sequences of three subunits included in group G were determined and the phylogenetic trees were produced using computer programs of parsimony and distance methods. Furthermore, I determined the partial amino acid sequence of one subunit included in group H which composed a heterodimer with a subunit

of group G and discuss the origin of the constituent monomers of the hemocyanin dimers.

2.2. Materials and Methods

2.2.1. Materials

The subunits, AKd1a, LTd1a, MGd1a and MGd1b were used for amino acid sequence analyses, as representatives of the constituent monomers of dimers. Preparation of hemocyanin samples was described in "Materials and Methods" of part one in this thesis.

2.2.2. Methods

2.2.2.1. Purification of constituent monomers of hemocyanin dimers

The hemocyanin samples were dialyzed against 50 mM Tris, 10 mM EDTA (pH 9.6) to dissociate hemocyanin subunits for 12 h at 4 °C and filtered through pores of 0.45 μm in diameter. Then the filtered samples were passed through a column of HiLoad 16/60 Superdex 200 prep grade (Pharmacia Biotech) to separate hemocyanin dimers from hemocyanin monomers and oligomers. Elution with 50 mM Tris, 10 mM EDTA (pH 9.6) was carried out at a flow rate of 0.5 ml per minute at room temperature.

The hemocyanin dimers in eluted fractions were electrophoretically collected. The dimer band in the gel was cut out, homogenized and dialyzed against the SDS-sample buffer containing 25 mM Tris, 0.1% SDS and 0.1% 2-mercaptoethanol (pH 6.8), and then were re-electrophoresed into the SDS slab gel to separate the constituent monomers from the dimers. The constituent monomer bands in the SDS slab gel were cut out, and soaked in the buffer containing 20 mM Tris and 1% SDS (pH 8.0) for 2 days at room temperature. After the extract containing constituent monomers were filtered to purge fragments of gel, the constituent monomers were precipitated by trichloroacetic acid precipitation (Stone *et*

al., 1989).

2.2.2.2. Chemical and enzymatic cleavage

The constituent monomers dissolved in 7 M guanidine hydrochloride, 0.5 M Tris, 10 mM EDTA (pH 8.5) were denatured and completely reduced with dithiothreitol (DTT, DDT:protein = 1:1 (w/w)) under nitrogen gas at 37 °C for 2 h. After cooling to room temperature, iodoacetamide (2 moles/mole of DTT) was added in the solution of reduced constituent monomers. The reaction was allowed to continue for 30 min in the dark. The mixture was desalted by gel filtration using Fast Desalting PC 3.2/10 (Pharmacia Biotech) in SMART system (Pharmacia Biotech), and freeze-dried. Freeze-dried constituent monomer peptides were dissolved to a concentration of 1% (w/v) in 70% formic acid. Cyanogen bromide (CNBr) dissolved in 70% formic acid was added to this solution (CNBr:protein = 50:1 (by mass)) and the reaction proceeded at 4 °C for 24 h in the dark (Yokota and Riggs, 1984). The reaction was terminated by addition of 10 volumes of water. On the other hand, digestion of the reduced proteins with lysyl endopeptidase (E/S = 1:100 (mol/mol)) was performed in 0.1M NH_4HCO_3, containing 2 M urea at 37 °C for 12 h (Stone *et al.*, 1989).

Large CNBr fragments were digested with V8 protease from *Staphylococcus aureus* in 4 M Urea, 50 mM NH_4HCO_3, pH 7.8 at a substrate/enzyme ratio of 50/1 (mol/mol) for 18 h at 37 °C. Digestion of large lysyl endopeptidase-digested fragments with trypsin was performed in 2 M Urea, 50 mM Tris-HCl, pH 8.0 at a substrate/enzyme ratio of 100/1 (mol/mol) for 12 h at 37 °C (Stone *et al.*, 1989).

2.2.2.3. Peptide separation

The CNBr fragments dissolved in 70% formic acid and the resulting peptides by enzymatic digestion were separated by reverse-phase chromatography using Sephasil C18 SC 2.1/10 (Pharmacia Biotech) or μRPC C2/C18 SC 2.1/10 (Pharmacia Biotech) in SMART system. The column was equilibrated with water containing 0.065% trifluoroacetic acid and the chromatography with a linear gradient from 0% to 60% of acetonitrile containing 0.055% trifluoroacetic was performed at flow rate of 50 μl/min. The effluent was monitored at 210 or 214 nm.

2.2.2.4. Amino acid sequence determination

A piece (5×5 mm) of PVDF membrane was activated for 1 min. in 100% methanol and transferred to water. In order to bind peptides, the piece was soaked in a fraction containing peptides at 4 °C for 24 h on the shaker. The piece binding the peptides was mounted in the reaction chamber of a protein sequencer. Sequences determination was performed with Procise 492 gas phase sequencer.

2.2.2.5. Computer analysis of aligned sequences

Sequences aligned by hand were analyzed using the PHYLIP 3.5c software package (Felsenstein, 1996), with gaps (deletion) considered as "missing data". The maximum parsimony (MP) analysis (Fitch, 1971) was done with PROTPERS of PHYLIP package. The neighbor-joining (NJ) analysis (Saitou and Nei, 1987) was done with NEIGHBOR of the package after pairwise sequence distances among all sequences were calculated with

PROTDIST using the Dayhoff PAM matrix. The reliability of the trees was tested by 1000 bootstrap replicates with the program SEQBOOT (Felsenstein, 1985).

2.3. Results

2.3.1. Amino acid sequence of AKd1a from *Atypus karschi*

The fragments obtained by CNBr cleavage (CNBr fragments) of AKd1a and the fragments obtained by digestion with lysyl endopeptidase (lysyl endopeptidase-digested fragments) of AKd1a were separated by reverse-phase chromatography, respectively. The chromatograms of the CNBr fragments and lysyl endopeptidase-digested fragments are shown in Figure 21A and B, respectively. One large CNBr fragment, CB6, was subdigested with V8 protease. The resulting peptides were separated by reverse-phase chromatography and analyzed their amino acid sequences. However, sequencing of those peptides presented no useful data. In the present study, sequencing of peptides in the fractions numbered in Figure 21A and B presented the sequences for the first 5-54 amino acid residues.

The sequences for individual peptides were aligned with the sequence of *A. californicum* hemocyanin subunit ACb (Sonner *et al.*, 1990) which is orthologous with AKd1a (Fig. 22). Sequencing of subunit AKd1a resulted in the determination of 424 amino acid residues.

2.3.2. Amino acid sequence of LTd1a from *Latouchia typica*

Elution profiles of CNBr fragments and lysyl endopeptidase-digested fragments of LTd1a by reverse-phase chromatography are shown in Figure 23A and B, respectively. Sequencing of peptides in the fractions numbered in Figure 23A and B presented the sequences for the first 3-32 amino acid residues.

These sequences for individual peptides were aligned with the sequence of *A. californicum* subunit ACb (Sonner *et al.*, 1990) which is orthologous with LTd1a (Fig. 24). Sequencing of subunit LTd1a resulted in the determination of 274 amino acid residues.

2.3.3. Amino acid sequence of MGd1a from *Macrothele gigas*

Elution profiles of CNBr fragments and lysyl endopeptidase-digested fragments of MGd1a by reverse-phase chromatography are shown in Figure 25A and B, respectively. Sequencing of peptides in the fractions numbered in Figure 25A and B presented the sequences for the first 7-41 amino acid residues.

Two large CNBr fragments, CB11 and CB12 were subdigested with V8 protease. The resulting peptides were separated by reverse-phase chromatography using Sephasil C18 SC 2.1/10 (Pharmacia Biotech) (results not shown). The amino acid sequence of one peptide CB11-V1 obtained by V8 protease-subdigestion of a fragment CB11 was determined for the 7 amino acid residues. However, sequences of peptides obtained by V8 protease-subdigestion of a fragment CB12 were ambiguous.

These sequences for individual peptides including N-terminal amino acid sequence were aligned with the sequence of *A. californicum* subunit ACb (Sonner *et al.*, 1990) which is orthologous with MGd1a (Fig. 26). Sequencing of subunit MGd1a resulted in the determination of 357 amino acid residues.

2.3.4. Amino acid sequence of MGd1b from *Macrothele gigas*

The chromatograms of CNBr fragments and lysyl endopeptidase-digested fragments of

MGd1b are shown in Figure 27A and B, respectively. Sequencing of peptides in the fractions numbered in Figure 27A and B presented the sequences for the first 6-46 amino acid residues.

Two large CNBr fragments, CB8 and CB9 were subdigested with V8 protease, and the resulting peptides were separated by reverse-phase chromatography using Sephasil C18 SC 2.1/10 (Pharmacia Biotech) (results not shown). The amino acid sequences of two peptides obtained by subdigestion of CB8 (CB8-V1 and V2) were analyzed. Sequencing of one resulting peptide of CB9 presented no useful sequence data, because the amino acid sequence was completely overlapped with that of CB9 (results not shown). Two lysyl endopeptidase-digested fragments (K4 and K9) were subdigested with trypsin. The resulting peptides of tryptic subdigestion of fragments K4 and K9 were separated by reverse-phase chromatography using Sephasil C18 SC 2.1/10 (Pharmacia Biotech). However, sequences of the analyzed tryptic peptides of K4 and K9 were completely overlapped with known sequences of K4 and K9, respectively (results not shown).

These sequences for individual peptides were aligned with the sequence of *A. californicum* subunit ACb (Sonner *et al.*, 1990) (Fig. 28). Sequencing of subunit MGd1b resulted in the determination of 331 amino acid residues.

2.4. Discussion

2.4.1. Phylogenetic analysis of mygalomorph spiders

For phylogenetic study of animals, it is necessary to analyze the orthologous molecules or genes. The eight groups of orthologous subunits are classified from the N-terminal sequence comparison (Table 1), and the subunits included in groups G and H are available for making evolutionary tree of the mygalomorph spiders because of their wide distribution in the Mygalomorphae (Fig. 19C).

The heterodimers of *A. karschi*, *L. typica* and *M. gigas* are made up by the constituent monomers of groups G and H, and the constituent monomer ACb of heterodimer bc in *A. californicum* (Markl *et al.*, 1979b) is a member of group G. Therefore, it is thought that another constituent monomer ACc is a member of group H. In fact, the partial amino acid sequence of ACc (Schartau *et al.*, 1986) showed some similarities to the sequence of MGd1b, but is uninformative for phylogenetic analysis. The monophyly of Antrodiaetidae plus Atypidae were strongly supported by Raven (1985) and Goloboff (1993) (Fig. 20). Thus, in the present study, although the subunits of group G were not found in Antrodiaetidae, the phylogenetic relationships of the mygalomorph spiders was investigated based on the amino acid sequences of the subunits included in groups G.

Figure 29 shows the MP tree and NJ tree produced by computer programs using amino acid sequences of the subunits included in group G (AKd1a, LTd1a, MGd1a and ACb) and known sequences of six chelicerate and one crustacean hemocyanin subunits. For phylogenetic analysis, amino acid residues in 171 sites aligned by hand were available.

The MP program produced two most parsimonious trees and the strict consensus tree of them is shown in Figure 29A.

Although the MP tree does not coincide with the NJ tree (Fig. 29B), both trees support the monophyly of the subunits of group G and the monophyly of chelicerate hemocyanin subunits with high bootstrap values. Furthermore, these trees show the monophyly of subunits LTd1a, MGd1a and ACb, which were found in the avicularioid spiders (Goloboff, 1993). Because the subunits of group G are orthologous subunits descended from a subunit in a common ancestral species, the relationships among subunits of group G reflect the evolutionary relationships among these four mygalomorph species, that is, *A. karschi*, *L. typica*, *M. gigas* and *A. californicum*. It is suggested from the two trees that *L. typica* of Ctenizidae, *M. gigas* of Hexathelidae and *A. californium* of Theraphosidae are closer to each other than to *A. karschi* of Atypidae.

In the higher classifications of the mygalomorph spiders, Goloboff (1993) did not agree with Raven (1985) about the position of the group Rastelloidina including family Ctenizidae (Fig. 20). The phylogenetic analysis based on amino acid sequences of hemocyanin subunit supports the monophyly of *L. typica* of Ctenizidae, *M. gigas* of Hexathelidae and *A. californicum* of Theraphosidae, though in the two trees (Fig. 29) the relationship among these spiders is different. This is congruent with the classification proposed by Goloboff (1993) in which Ctenizidae, Hexathelidae and Theraphosidae were included in one group Avicularioidea.

In this work, based on molecular sequence data, the phylogenetic relationship of the mygalomorph spiders was investigated for the first time. The first results of molecular

phylogeny of the mygalomorph spiders support the Goloboff's classification of the mygalomorph spiders. His classification is also supported 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) (Fig. 19).

2.4.2. The origin of constituent monomers of hemocyanin dimers

In order to analysis the evolutionary relationships among the constituent monomers of the mygalomorph hemocyanin dimers, the phylogenetic trees were produced using amino acid residues in available 233 sites of MGd1a, MGd1b, ACb and known six chelicerate and one crustacean subunits. The trees from MP (Fig. 30A) and NJ (fig,. 30B) analyses supported the monophyly of the subunits of groups G (MGd1a and ACb) and H (MGd1b), though the difference between them arose. The monophyly of chelicerate hemocyanin subunits was also supported. The monophyly of the constituent monomers of groups G and H suggests that these constituent monomers of heterodimers in the mygalomorph hemocyanins were originated from a common ancestral subunit.

The N-terminal sequences of the constituent monomers of a scorpion (Sugita *et al.*, 1999) and a whipscorpion (Kuwada *et al.*, 2001) hemocyanin dimers showed the orthologous relationship with subunits of group G in the mygalomorph hemocyanin dimers (Kuwada *et al.*, 2001). Therefore, it is thought that these constituent monomers of arachnid hemocyanin dimers are originated from a common ancestral subunit and conserved in these animals.

The phylogenetic trees in Figures 29 and 30 support the monophyly of chelicerate hemocyanin subunits including the constituent monomers of hemocyanin dimers, and the monophyly of chelicerate hemocyanin subunits has been shown in some studies (Beintema *et al.*, 1994; Burmester and Scheller, 1996; Jamroz *et al.*, 1996; Burmester, 1999; Hughes, 1999; Terwilliger *et al.*, 1999). Thus it is clear that the gene duplication which brought subunits of groups G and H happened in a chelicerate lineage after the divergence of two lineages of chelicerates and crustaceans. The hemocyanin dimers were also found in crustaceans (Murray and Jeffrey, 1974; Markl, *et al.*, 1979c), and Markl *et al.* (1981) concluded that the hemocyanin dimers of a crayfish, *Astacus leptodactylus*, function as a bridge between hexamers as well as those of chelicerates. Even if the origin of the constituent monomers of crustacean hemocyanin dimers cannot be revealed, it is concluded that the function of the hemocyanin dimer as a "linker" molecule were acquired independently in the two lineages of chelicerates and crustaceans.

General Discussion

When a cladogram (divergence pattern) is produced using molecular sequences, a species tree have to be distinguished from a gene tree. The relation between species and gene trees is diagrammed in Figure 31. If, through ill luck, genes xA and xC were isolated respectively from species A and C and gene yB from species B, the inferred tree (Fig. 31B) would not be congruent with the species tree (Fig. 31A). If a set of orthologous genes xA, xB and xC (or yA, yB and yC) is used, a species tree can be produced (Fig. 31C). Therefore, to investigate the evolutionary history of animals based on molecular sequence data, the orthologous molecules originated from a common ancestral gene have to be prepared for sequence analysis. It was shown that the N-terminal amino acid sequence is useful to identify the orthologous hemocyanin subunits between species having many homologous subunits (Takasu and Sugita, 1997; Sugita and Murayama, 1998; Sugita *et al.*, 1999). In this study, I reported, for the first time, phylogenetic relation of animals, the mygalomorph spiders, based on sequence data of orthologous hemocyanin subunits identified from the N-terminal amino acid sequence comparison. And phylogenetic trees of the mygalomorph spiders produced by computer programs supported the higher classification proposed by Goloboff (1993).

The distribution patterns of the hemocyanin monomers (Fig. 19) also supported the higher classification proposed by Goloboff (1993). Although the duplication and deletion of hemocyanin subunits give some confusion in evolutionary studies of hemocyanin molecules, the distribution patterns of the hemocyanin subunits are considered as a phylogenetic marker. In fact, although no common orthologous subunit is found between

Araneidae and Pisauridae spiders of Araneomorphae, orthologous hemocyanin subunits are shared in araneid spiders but not in a pisaurid spider (Takasu and Sugita 1997). Furthermore, it was inferred from N-terminal sequence comparison that after the divergence of two lineages of American and Asian horseshoe crabs, duplications of hemocyanin subunits happened in the American horseshoe crab lineage (Sugita and Suzuki 1998). These reports support that the restricted distribution of hemocyanin subunits to particular taxa is results from duplication and deletion happened independently in each of animal lineages. Therefore, comparison of the distribution patterns of orthologous hemocyanin subunits is a good method for taxonomic study on the animals having hemocyanin subunits.

The mygalomorph spiders have 24-mer (4×hexamer) hemocyanin and the advanced spiders, araneomorphs, have 12-mer (2×hexamer) hemocyanin (Markl *et al.*, 1979a; Ellerton *et al.*, 1983). From the evidence that seven different subunits compose 24-mer in the mygalomorph spider *A. californicum* hemocyanin and two different subunits compose 12-mer in the araneomorph spider *Cupiennius salei* hemocyanin, Markl *et al.* (1986) indicated that the 24-mer hemocyanin composed of seven subunits changed to 12-mer hemocyanin composed of two subunits during the evolution of spiders. Furthermore, from the evidence that the araneomorph spiders have tracheae which supply the inner tissues directly with oxygen, Hausdorf (1999) suggested that the changes in hemocyanin compositions were due to a decrease of the selective pressure by the evolution of tracheae in the araneomorph spiders. However, it is clear that the duplication and the loss of the hemocyanin subunits, which brought the varieties of subunit numbers, often occurred during the evolution of the mygalomorph spiders which have 24-mer hemocyanin and not

tracheae. Therefore, it is thought that the increase or decrease of the number of the hemocyanin monomer subunit are related with neither the change of hemocyanin composition nor the evolution of tracheae. Reese and Mangum (1994) revealed that there was no relationship between intrinsic respiratory properties and subunit composition of hemocyanin, 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 animals having hemocyanins. Therefore, the changes of hemocyanin might have been allowed within the limit of survival of these animals, and the resulting distribution patterns of the subunits allowed me to classify the mygalomorph spiders.

On the other hand, the constituent monomers of the hemocyanin dimers, that is the subunits of groups G and H, were conserved in the mygalomorph spiders except *A. roretzi* of which hemocyanin dimers are homodimers of group H subunits. And the hemocyanin dimers in a scorpion and a whipscorpion were composed of the orthologous subunits of group G (Kuwada *et al.*, 2001), suggesting that the hemocyanin dimers have been conserved during the evolution of arachnids. However, the duplication of the dimer subunits was also allowed in the lineage of *M. gigas* because of no harm to the spiders. But the loss of dimer subunits was not allowed under functional restraint as a linker of two hexamers and the dimer subunits were conserved widely in the evolutionary history of these animals, allowing me to make species tree of the mygalomorph spiders.

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Table

Table 1.
Comparison of N-terminal sequences of mygalomorph hemocyanin subunits within each group.

Group	Subunit	N-terminal sequence	% similarity					
		-6 1 11 21	AR4					
A	AR4	SPQAHGTVHAKQLKVNALFEHLTALTGHVIP						
	AY4	SPQAHGSVHEKQLKVNGLFEHLTALTRHVI	87					
		-5 1 11 21	AK2	AK3	AK4			
	AK2	TPKAQTVHEKQLRVNXDFA						
B	AK3	TPKAQTVHEKQLRVNSLFAHL	94					
	AK4	TPDAQTVVEKLLR	77	77				
	CS5	TPKAQTVHEKQLRVNSLFGHXTAXTHXQIP	89	95	77			
		1 11 21 31 41	LT2	UF1	MG1	MY1	MY2	MY3
	LT2	TILHDKQVQVLKLFEKLSVAAVQ-KVPEXQV						
	UF1	TILHDKQVRVLKIFERLSVAX-GEAXPIKE	69					
C	MG1	TILHDKQVQVLKLFEKLSVAATGHATDAN-IDARLKHLTHL	72	68				
	MY1	TILHDKQVQVLKLFEKLSVA	100	85	100			
	MY2	TILHDKQVQVLKLFEKLSVAATGNH-HGDDIDARLKNLTHLGPT	72	64	83	100		
	MY3	TILHDKQVQVLKLFEKLSVAATGNH-HGNDIDARLKNLTHL	72	64	83	100	98	
	ACa	TILHDKQVQALKLFEKLSVAATGEPVPADQIDERLRNITTLGPN	77	68	71	95	73	73
		1 11 21	LT1	MY4				
	LT1	PDKQKQLRVINLFQHMMXI						
D	MY4	PEKQKQLRVISLFEHMTSITKXLP	78					
	ACe	PDKQKQLRVISLFEHMTSINTPLP	83	87				
		1 11 21 31	LT3	LT4	MG3	MG4	MY6	
	LT3	TVKDKQRQILPLFEHLTSLTAGGLSHAD						
	LT4	TVKDKQRQILPLFEHLTSLTAGGLRHAD	96					
E	MG3	TVKDKQLEILPLFEHLTSLTGTGLPPEGRDHRLAKVGKLP	71	71				
	MG4	-VKDKQLEILPLFEHLTSLTGTGLPP	73	73	96			
	MY6	TVKDKQLEILPLFEHLTSLTGTGLPRDDR	75	75	90	92		
	MY7	TVKDKQLEILPLFEHLTSLTGTDLP	76	76	96	92	96	
		1 11 21	MG2					
F	MG2	GIPEKXAKLLALFEKLXNLTKE						
	MY5	GIHEKQVKLLALFEKLTSLTK	84					

Table 1. Continued

Group	Subunit	N-terminal sequence	% similarity				
		1 11 21 31	AK5	AKd1a	LTd1a	MGd1a	MGd2a
	AK5	-PAAEKQVRVLPFFQYASLTTR					
	AKd1a	SPAAEKQVRVLPFFQYASLTTKDKF	91				
G	LTd1a	PSTAEKQQRILPFFQFTSLSTKDKFGILVQRD	64	68			
	MGd1a	PSTAEKQQRILPFFQFTTLSTKDKFGILVQRDRRLA	62	64	97		
	MGd2a	PSTAEKQQRILPFFQFTTLSTKDKFGILVQRDRRLA	62	64	97	100	
	ACb	PSTAEKQVRILPFFQFTSLSTKXXXXXXXXXXXXLA	71	68	95	91	91
		-3 1 11	ARd1	ARd2	AKd1b	LTd1b	MGd1b
	ARd1	MPSDPSEKQIRLLSWFEHLS					
	ARd2	MPSDPSEKQIRLLSWFEHLS	100				
H	AKd1b	-SPDASEKQARLXSXFYH	63	63			
	LTd1b	-PSDANERQARLLXLP	53	53	58		
	MGd1b	-PHEANEKQAILLERF	47	47	54	57	
	MGd2b	-PHEANEKQAILLERFEHSL	47	47	53	57	100

Sequences data of *A. californicum* hemocyanin subunits ACa, ACb and ACe are from Voit and Feldmaier-Fuchs (1990), Sonner *et al.* (1990) and Schneider *et al.* (1983), respectively. 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 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. The subunit CS5 of group B is a hemocyanin monomer of *Calommata signatum* in family Atypidae. Because the subunits CS1-4 could not be separated sufficiently to be sequenced, the sequence of *C. signatum* subunit CS5 was shown only in this Table 1 as an orthologous sequence of group B.

Figures

Figure 1.

Native PAGE banding patterns of hemocyanin subunits of seven mygalomorph spiders, *A. roretzi* (*A. r.*), *A. yesoensis* (*A. y.*), *A. karschi* (*A. k.*), *L. typica* (*L. t.*), *U. fragaria* (*U. f.*), *M. gigas* (*M. g.*) and *M. yaginumai* (*M. y.*). The 7.5% acrylamide gels were prepared for electrophoresis of hemocyanins of the spiders except *M. yaginumai*. The 8.25 % acrylamide gel were prepared for *M. yaginumai* hemocyanin, because a monomer subunit (MY7) overlapped with a dimer subunit (MYd1) in the 7.5% acrylamide gel (see also Fig. 14). After electrophoresis, the gels were stained by CBB and the bands with arrowhead were also stained by rubeanic acid.

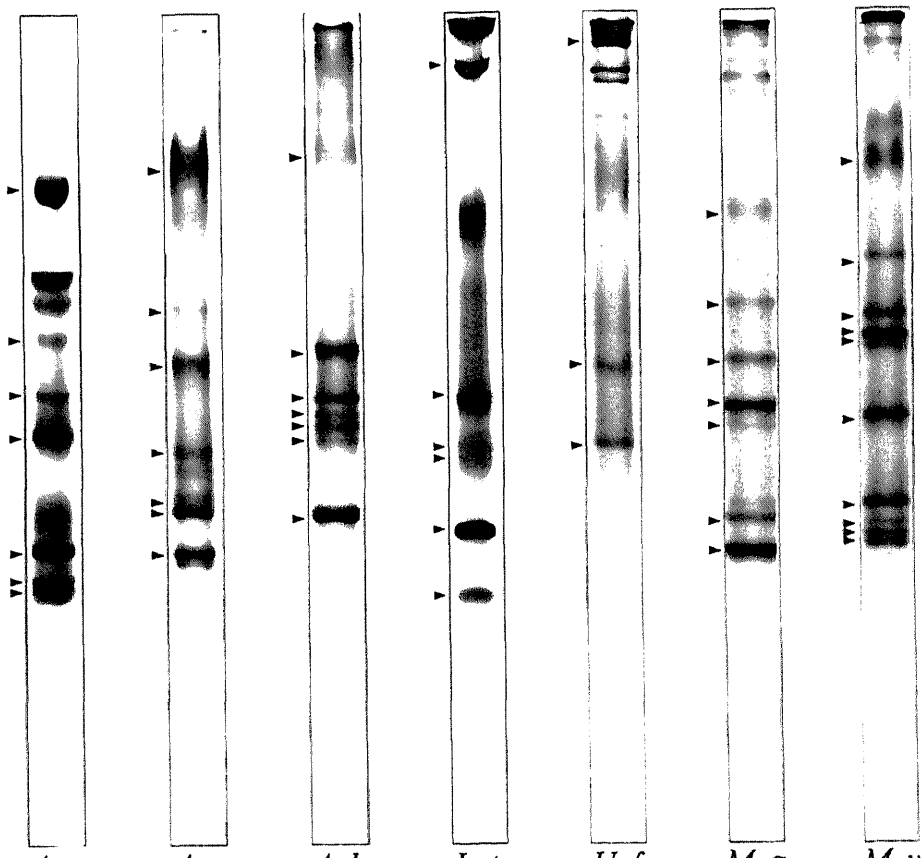

A. r.
A. y.
A. k.
L. t.
U. f.
M. g.
M. y.

Figure 2.

Native PAGE banding patterns of *A. roretzi* hemocyanin subunits in various acrylamide gel concentrations. The hemocyanin monomer bands (AR1-AR4) were numbered from the bottom to the top of the gels, and the hemocyanin dimer bands were named ARd1 and ARd2. The protein band with an asterisk is a hemocyanin oligomer band.

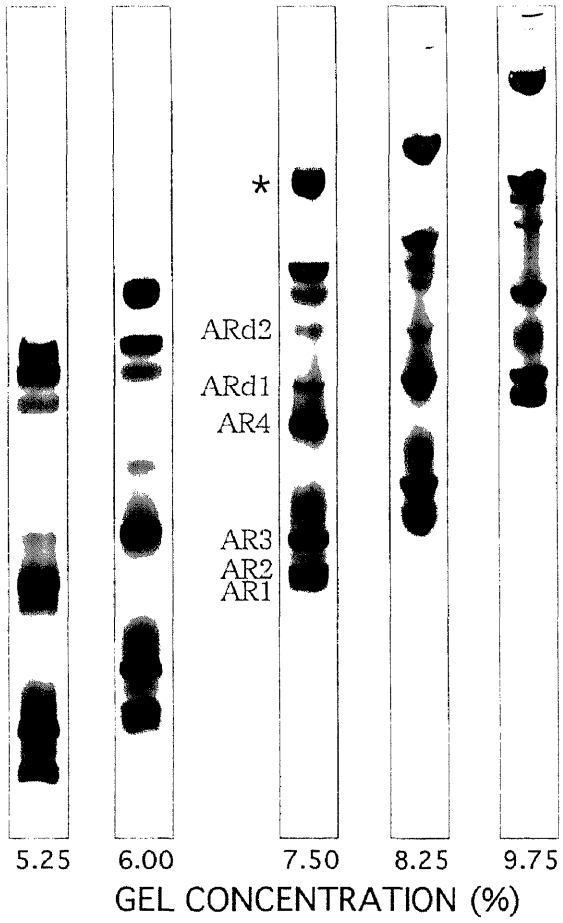

*
ARd2
ARd1
AR4
AR3
AR2
AR1
5.25
6.00
7.50
8.25
9.75
GEL CONCENTRATION (%)

Figure 3.

The effect of various gel concentrations on the mobility of hemocyanin monomer and dimer subunits in the *A. roretzi* hemocyanin. Hemocyanin monomer AR2 was separated between AR1 and AR3 and its slope was almost equal to those of AR1 and AR3. ●, hemocyanin monomer; ○, hemocyanin dimer; △, bovine serum albumin monomer; □, bovine serum albumin dimer; *, hemocyanin oligomer.

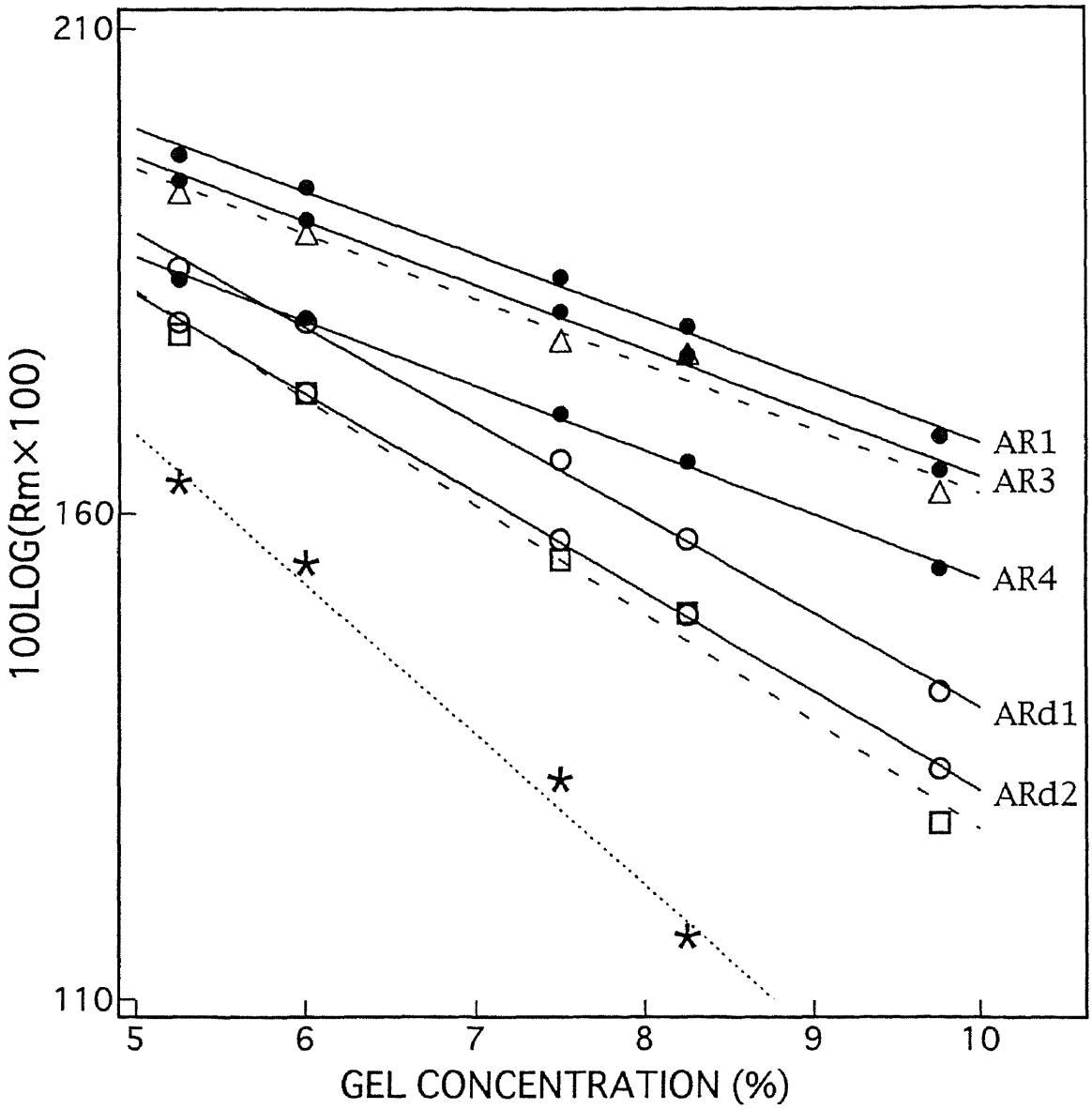

210
160
110
100LOG(Rm×100)
5
6
7
8
9
10
GEL CONCENTRATION (%)
AR1
AR3
AR4
ARd1
ARd2

Figure 4.

Native PAGE banding patterns of *A. yesoensis* hemocyanin subunits in various acrylamide gel concentrations. The hemocyanin monomer bands (AY1-AY4) were numbered from the bottom to the top of the gels, and the hemocyanin dimer bands were named AYd1 and AYd2. The protein band with an asterisk is a hemocyanin oligomer band.

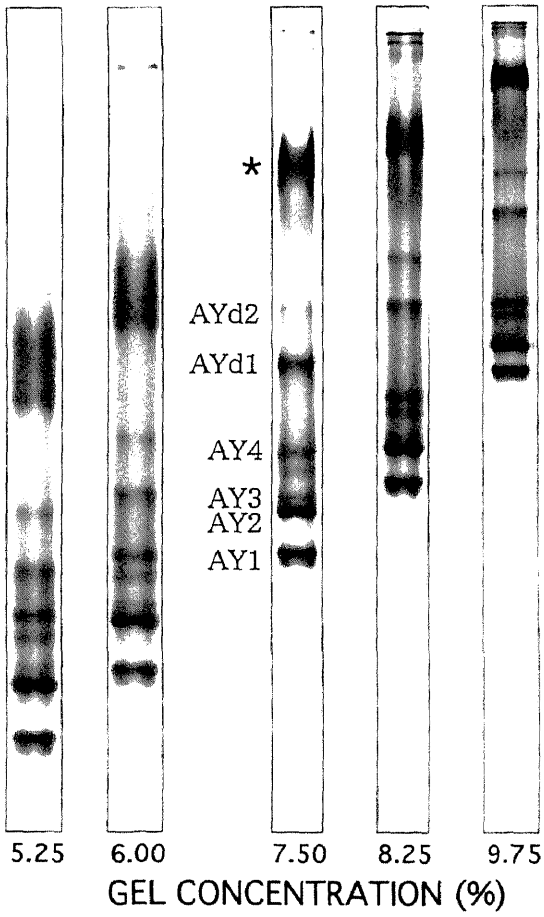

*
AYd2
AYd1
AY4
AY3
AY2
AY1
5.25
6.00
7.50
8.25
9.75
GEL CONCENTRATION (%)

Figure 5.

The effect of various gel concentrations on the mobility of hemocyanin monomer and dimer subunits in the *A. yesoensis* hemocyanin. Hemocyanin monomer AY2 was separated between AY1 and AY3 and its slope was almost equal to those of AY1 and AY3. Other explanations as in Fig. 3.

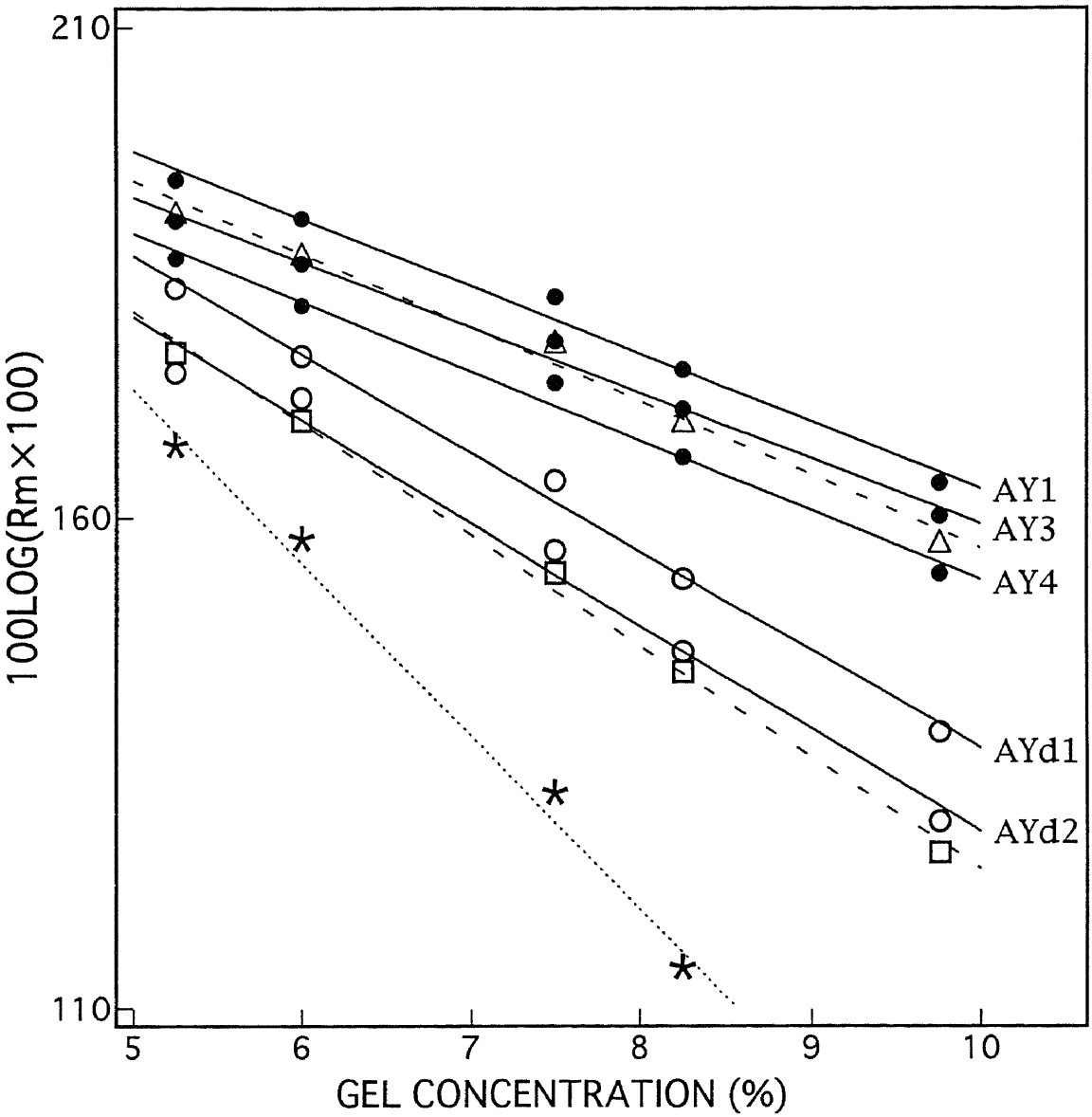

210
160
110
100LOG(Rm×100)
5
6
7
8
9
10
GEL CONCENTRATION (%)
AY1
AY3
AY4
AYd1
AYd2

Figure 6.

Native PAGE banding patterns of *A. karschi* hemocyanin subunits in various acrylamide gel concentrations. The hemocyanin monomer bands (AK1-AK5) were numbered from the bottom to the top of the gels, and the hemocyanin dimer band was named AKd1. The protein band with an asterisk is a hemocyanin oligomer band.

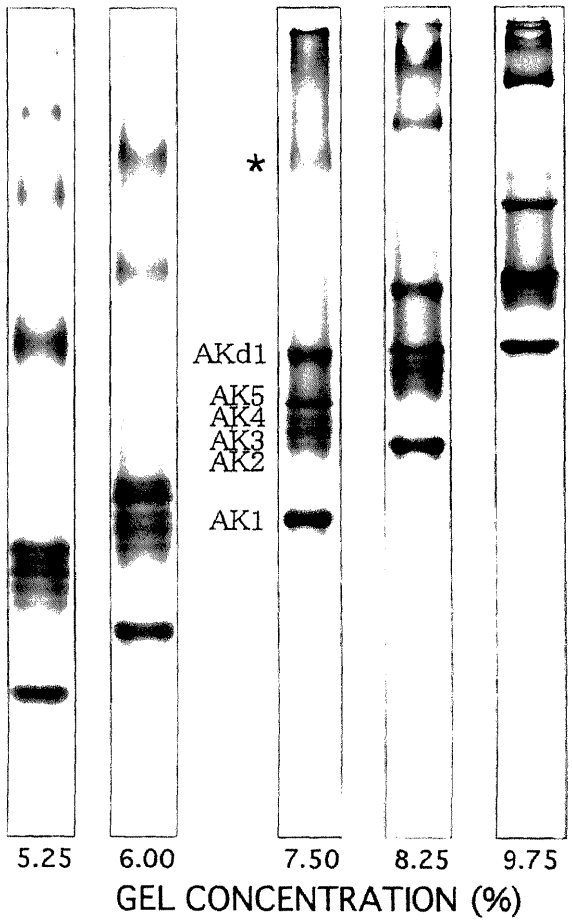

*
AKd1
AK5
AK4
AK3
AK2
AK1
5.25
6.00
7.50
8.25
9.75
GEL CONCENTRATION (%)

Figure 7.

The effect of various gel concentrations on the mobility of hemocyanin monomer and dimer subunits in the *A. karschi* hemocyanin. The subunits of AK3 and AK4 were separated between AK2 and AK5, respectively, and their slopes were almost equal to those of AK2 and AK5. Other explanations as in Fig. 3.

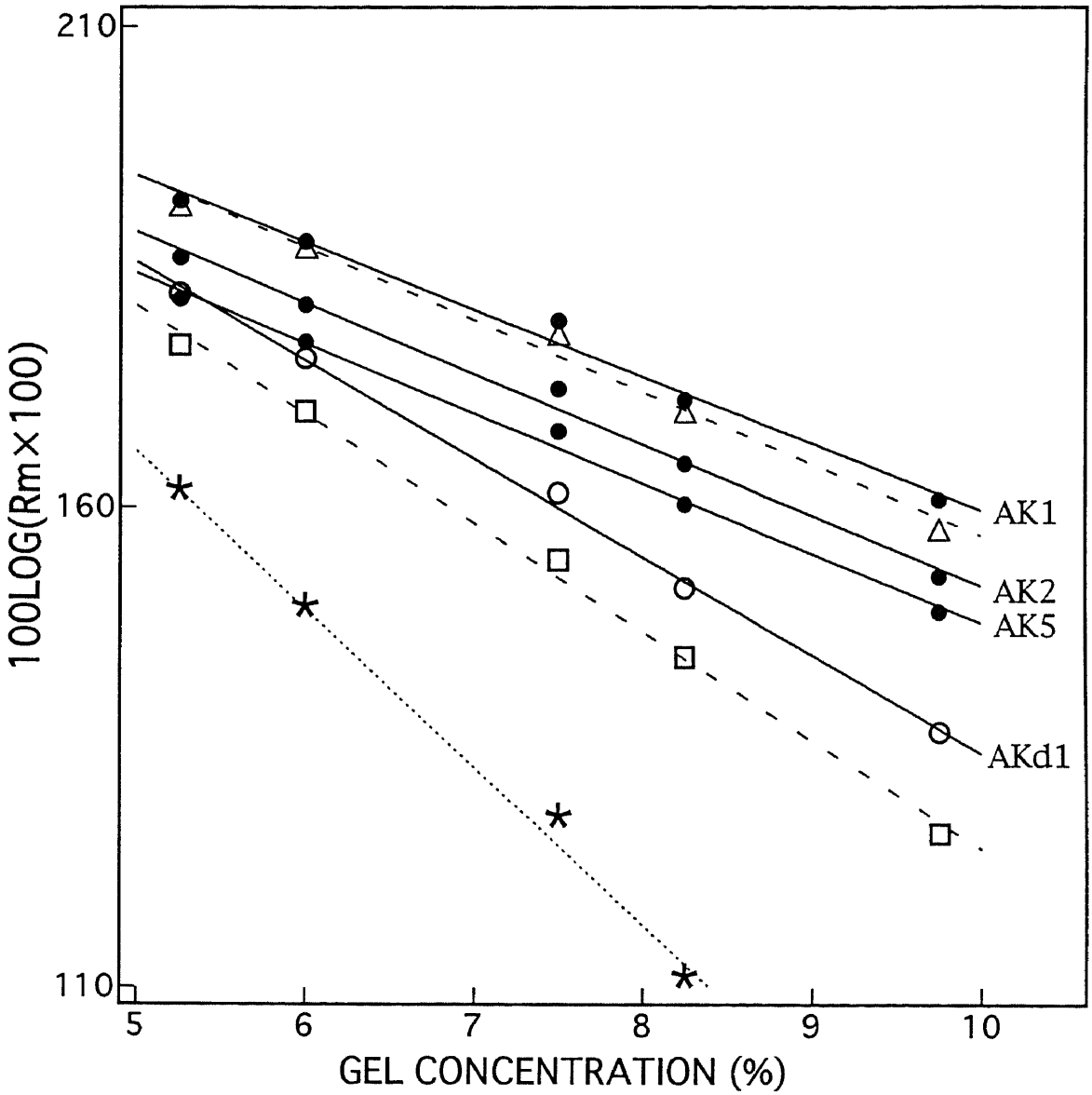

210
160
110
100LOG(Rm×100)
5
6
7
8
9
10
GEL CONCENTRATION (%)
AK1
AK2
AK5
AKd1

Figure 8.

Native PAGE banding patterns of *L. typica* hemocyanin subunits in various acrylamide gel concentrations. The hemocyanin monomer bands (LT1-LT4) were numbered from the bottom to the top of the gels, and the hemocyanin dimer band was named LTd1. The protein band with an asterisk is a hemocyanin oligomer band.

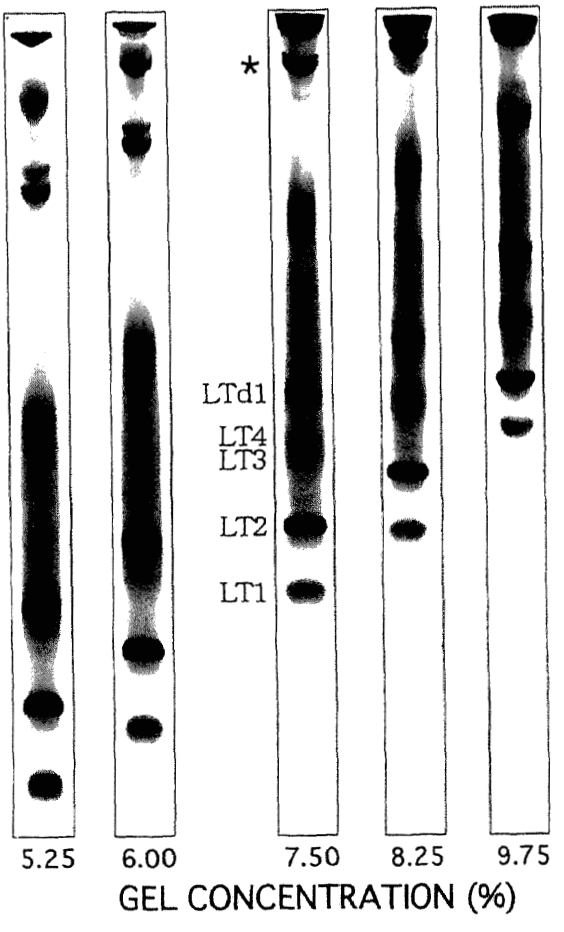

*
LTd1
LT4
LT3
LT2
LT1
5.25
6.00
7.50
8.25
9.75
GEL CONCENTRATION (%)

Figure 9.

The effect of various gel concentrations on the mobility of hemocyanin monomer and dimer subunits in the *L. typica* hemocyanin. Other explanations as in Fig. 3.

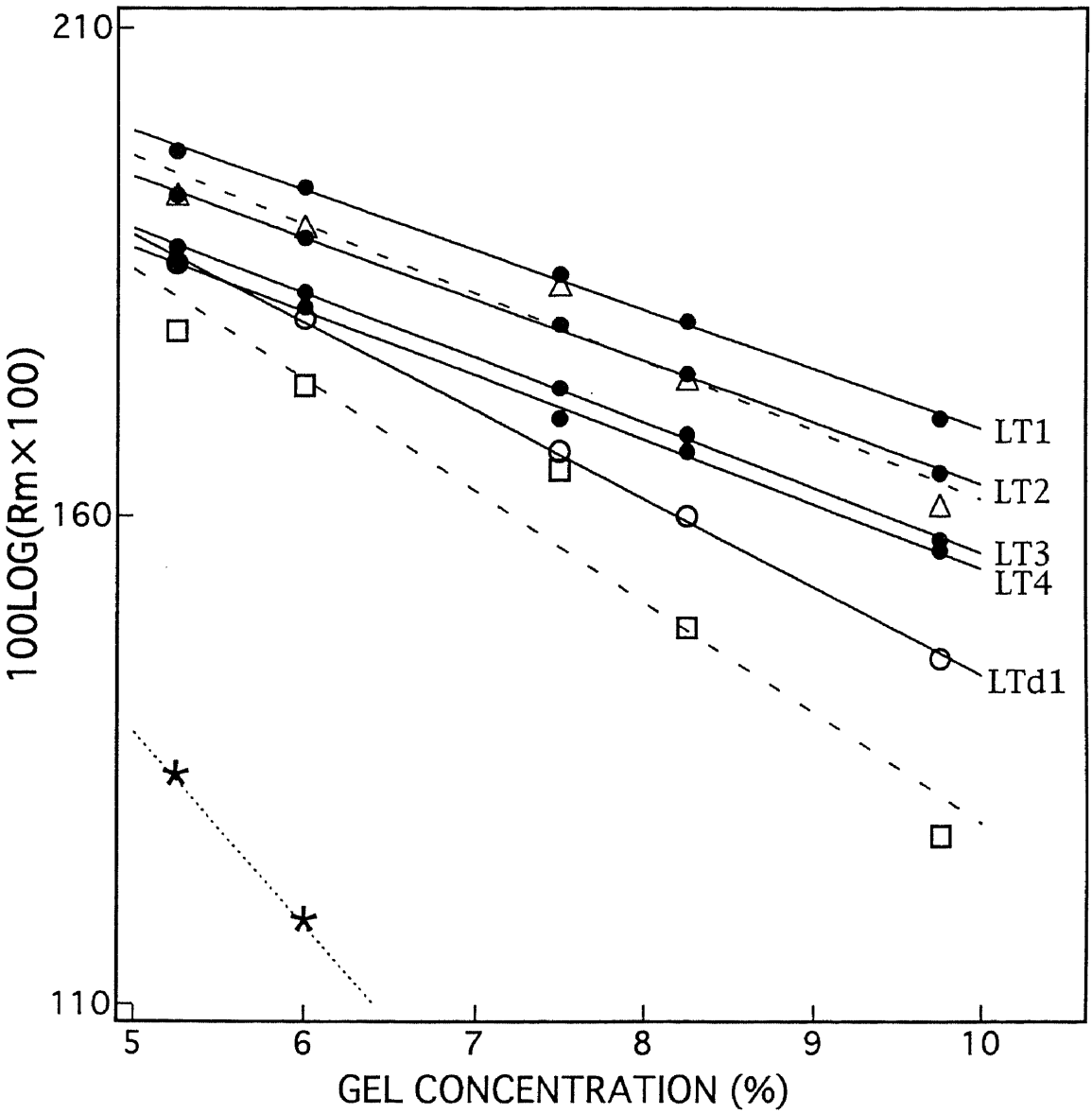

210
160
110
100LOG(Rm×100)
5
6
7
8
9
10
GEL CONCENTRATION (%)
LT1
LT2
LT3
LT4
LTd1

Figure 10.

Native PAGE banding patterns of *U. fragaria* hemocyanin subunits in various acrylamide gel concentrations. The hemocyanin monomer band and the dimer band were named UF1 and UFd1, respectively. The protein band with an asterisk is a hemocyanin oligomer band.

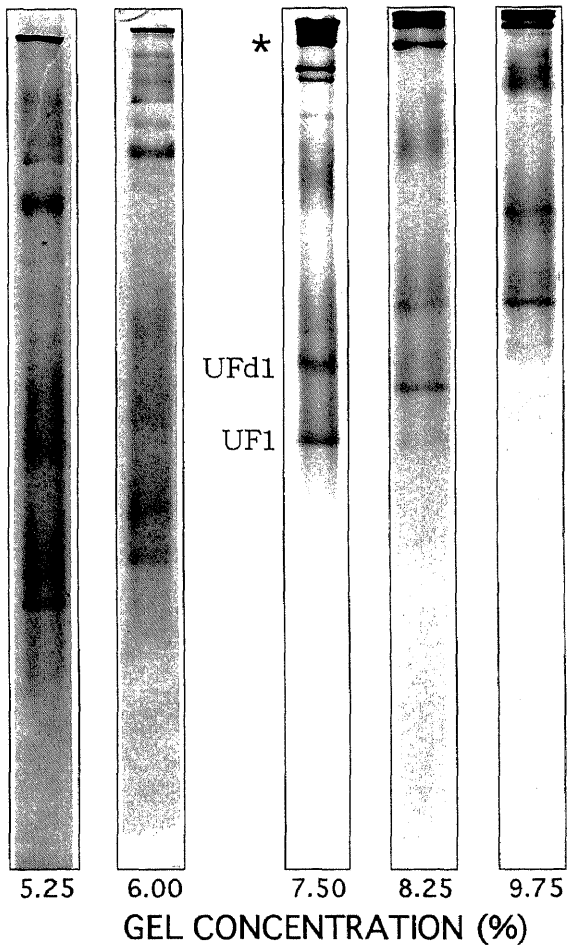

*
UFd1
UF1
5.25
6.00
7.50
8.25
9.75
GEL CONCENTRATION (%)

Figure 11.

The effect of various gel concentrations on the mobility of hemocyanin monomer and dimer subunits in the *U. fragaria* hemocyanin. Other explanations as in Fig. 3.

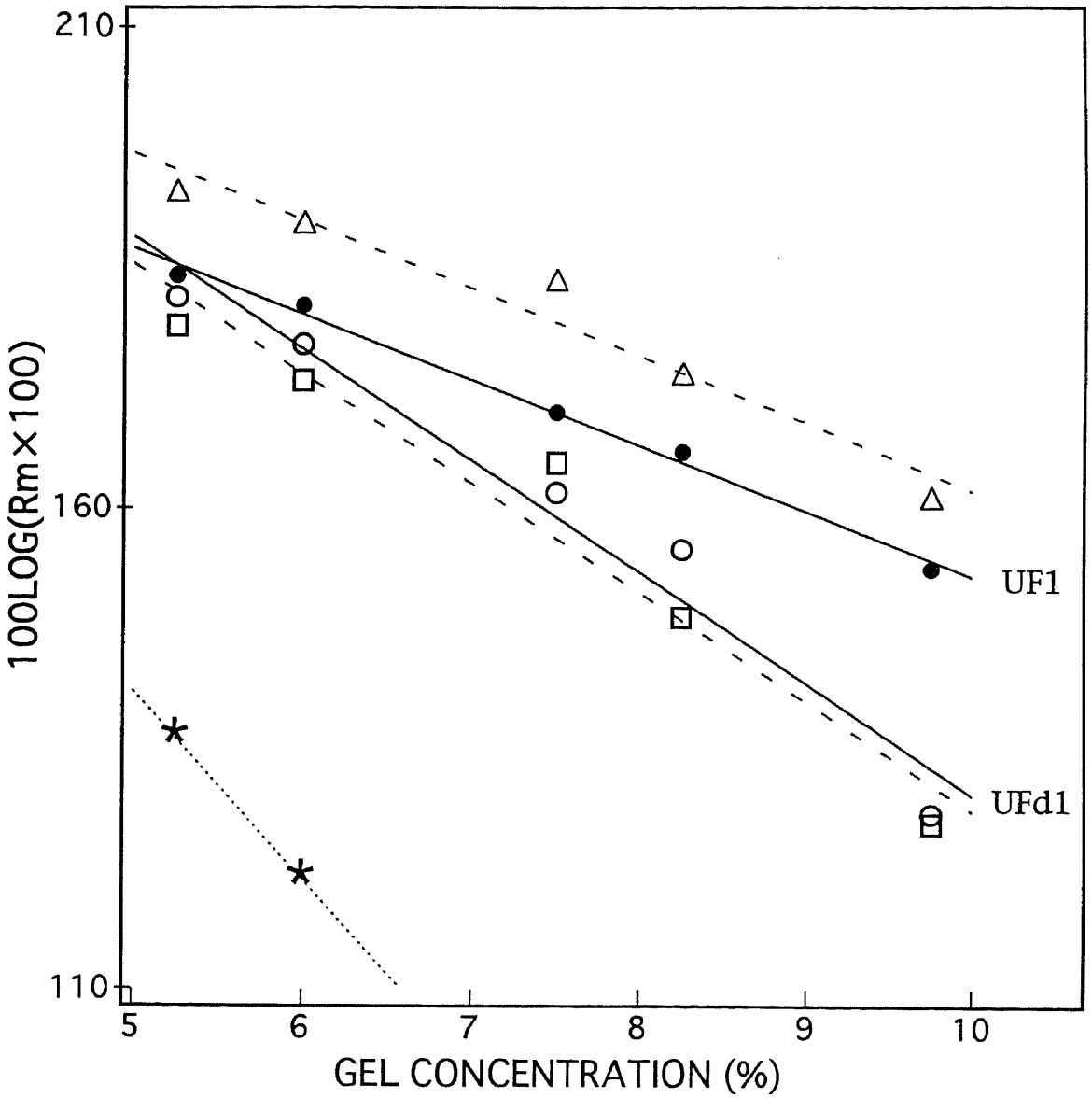

210
160
110
100LOG(Rm×100)
5
6
7
8
9
10
GEL CONCENTRATION (%)
UF1
UFd1

Figure 12.

Native PAGE banding patterns of *M. gigas* hemocyanin subunits in various acrylamide gel concentrations. The hemocyanin monomer bands (MG1-MG4) were numbered from the bottom to the top of the gels, and the hemocyanin dimer bands were named MGd1 and MGd2. The protein band with an asterisk is a hemocyanin oligomer band.

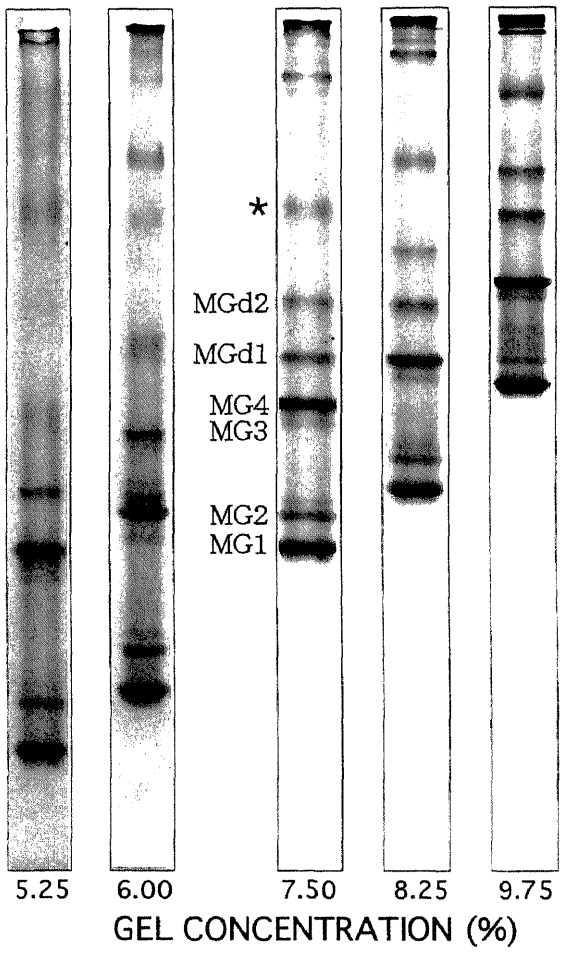

*
MGd2
MGd1
MG4
MG3
MG2
MG1
5.25
6.00
7.50
8.25
9.75
GEL CONCENTRATION (%)

Figure 13.

The effect of various gel concentrations on the mobility of hemocyanin monomer and dimer subunits in the *M. gigas* hemocyanin. Other explanations as in Fig. 3.

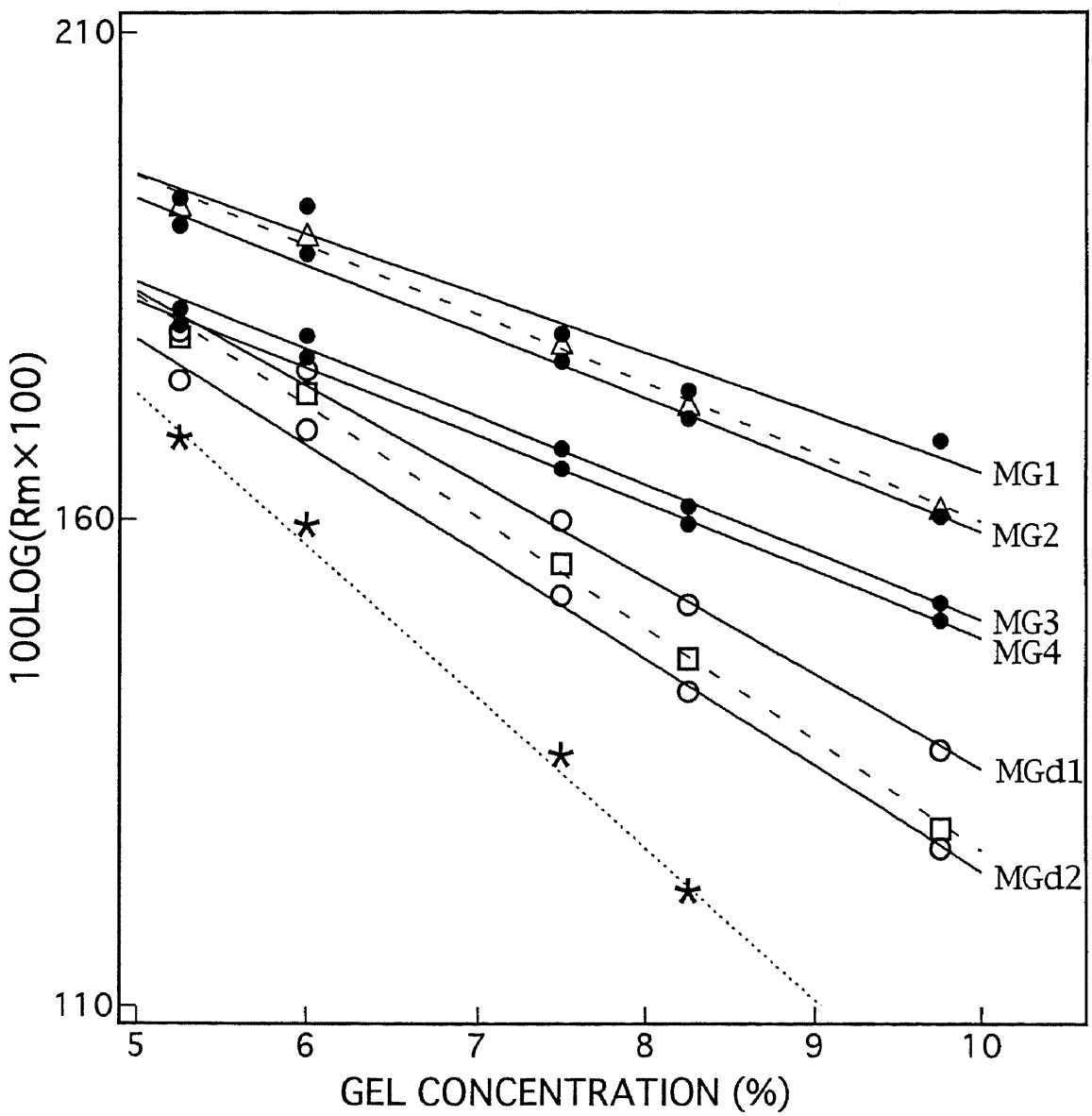

210
160
110
100LOG(Rm×100)
5
6
7
8
9
10
GEL CONCENTRATION (%)
MG1
MG2
MG3
MG4
MGd1
MGd2

Figure 14.

Native PAGE banding patterns of *M. yaginumai* hemocyanin subunits in various acrylamide gel concentrations. The hemocyanin monomer bands (MY1-MY7) were numbered from the bottom to the top of the gels, and the hemocyanin dimer bands were named MYd1 and MYd2. A monomer band (MY7) overlapped with a dimer band (MYd1) is denoted as 7&d1 on the left side of a column of the 7.5% acrylamide gel. The protein band with an asterisk is a hemocyanin oligomer band.

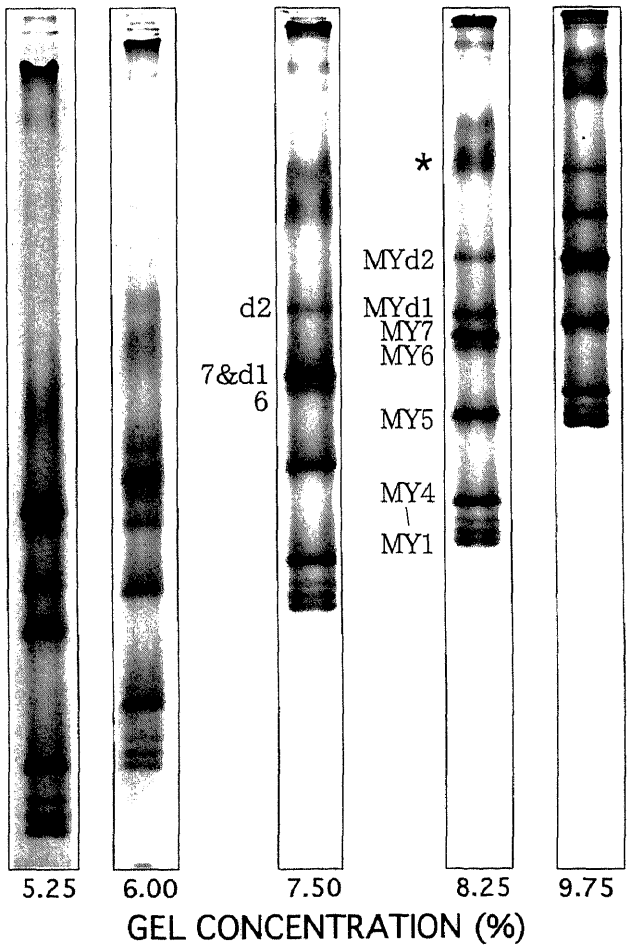

*
MYd2
d2
MYd1
MY7
MY6
7&d1
6
MY5
MY4
MY1
5.25
6.00
7.50
8.25
9.75
GEL CONCENTRATION (%)

Figure 15.

The effect of various gel concentrations on the mobility of hemocyanin monomer and dimer subunits in the *M. yaginumai* hemocyanin. The subunits of MY2 and MY3 were separated between MY1 and MY4, respectively, and MY6 was separated between MY5 and MY7. Their slopes were almost equal to those of bovine serum albumin monomer. Other explanations as in Fig. 3.

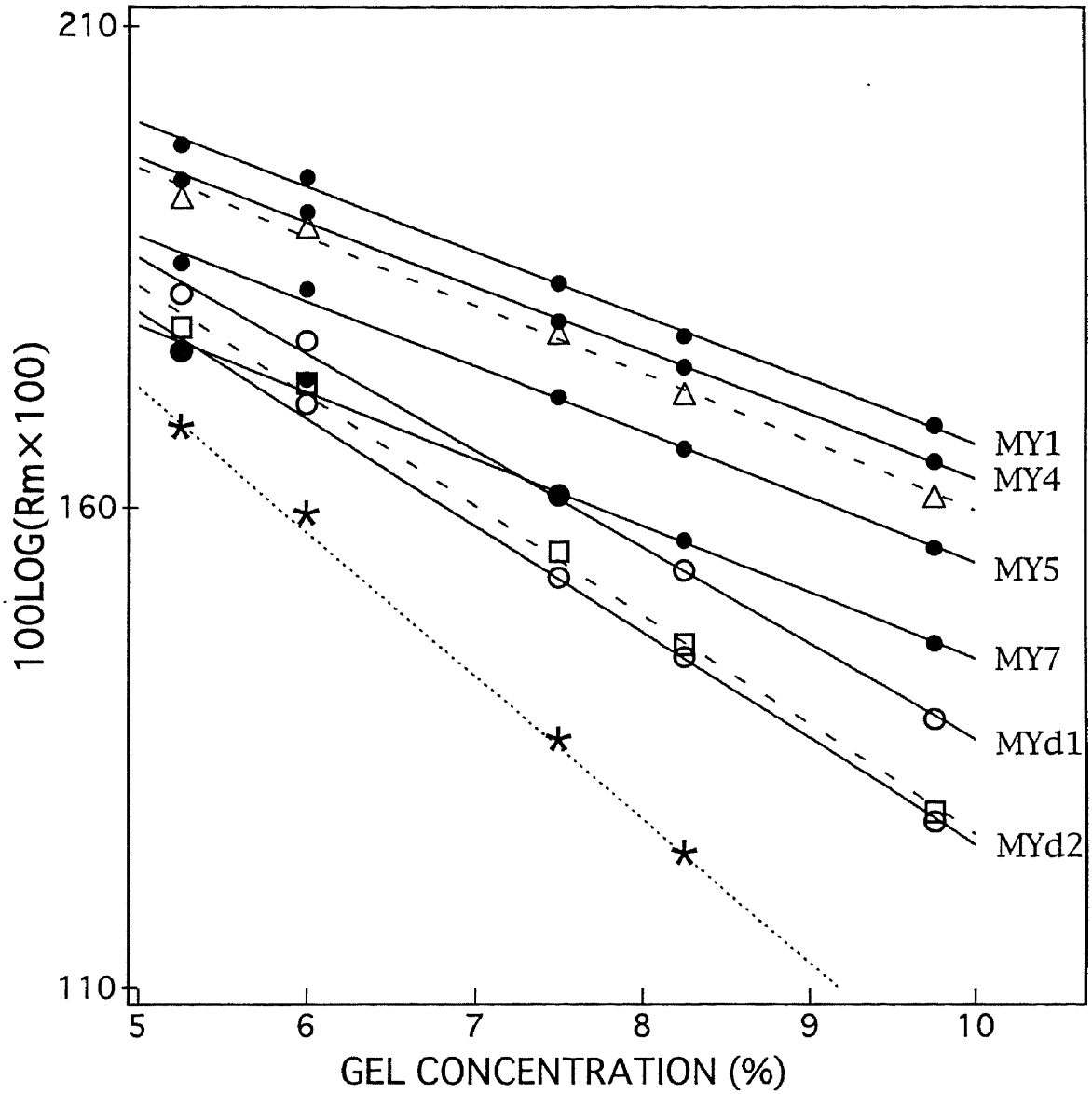
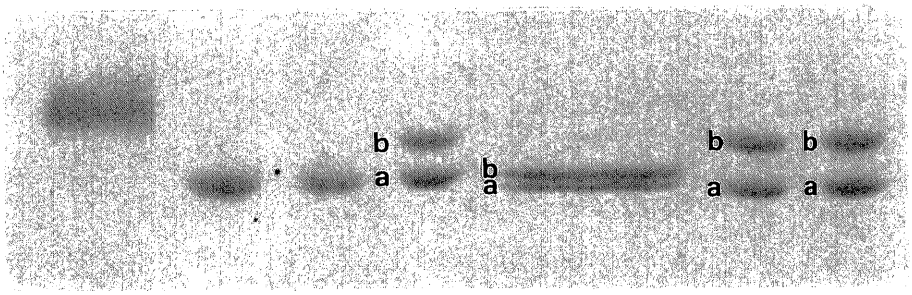

210
160
110
100LOG(Rm×100)
5
6
7
8
9
10
GEL CONCENTRATION (%)
MY1
MY4
MY5
MY7
MYd1
MYd2

Figure 16.

SDS PAGE banding patterns of constituent monomers of hemocyanin dimer subunits in a slab gel. BSA is bovine serum albumin monomer (84 kDa) in Bio-Rad's SDS-PAGE standards. ARd1 and d2, AKd1, LTd1 and MGd1 and d2 are hemocyanin dimer subunits, which were treated with the SDS-sample buffer, from *A. roretzi*, *A. karschi*, *L. typica* and *M. gigas*, respectively. The constituent monomers of lower molecular weight are denoted as "a" and those of higher molecular weight are denoted as "b".



BSA ARd1 ARd2 AKd1 LTd1 MGd1 MGd2

Figure 17.

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. gigas* (MG1-4) and *M. yaginumai* (MY1-7). 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 in positions 5, 6 and 13 are boxed.

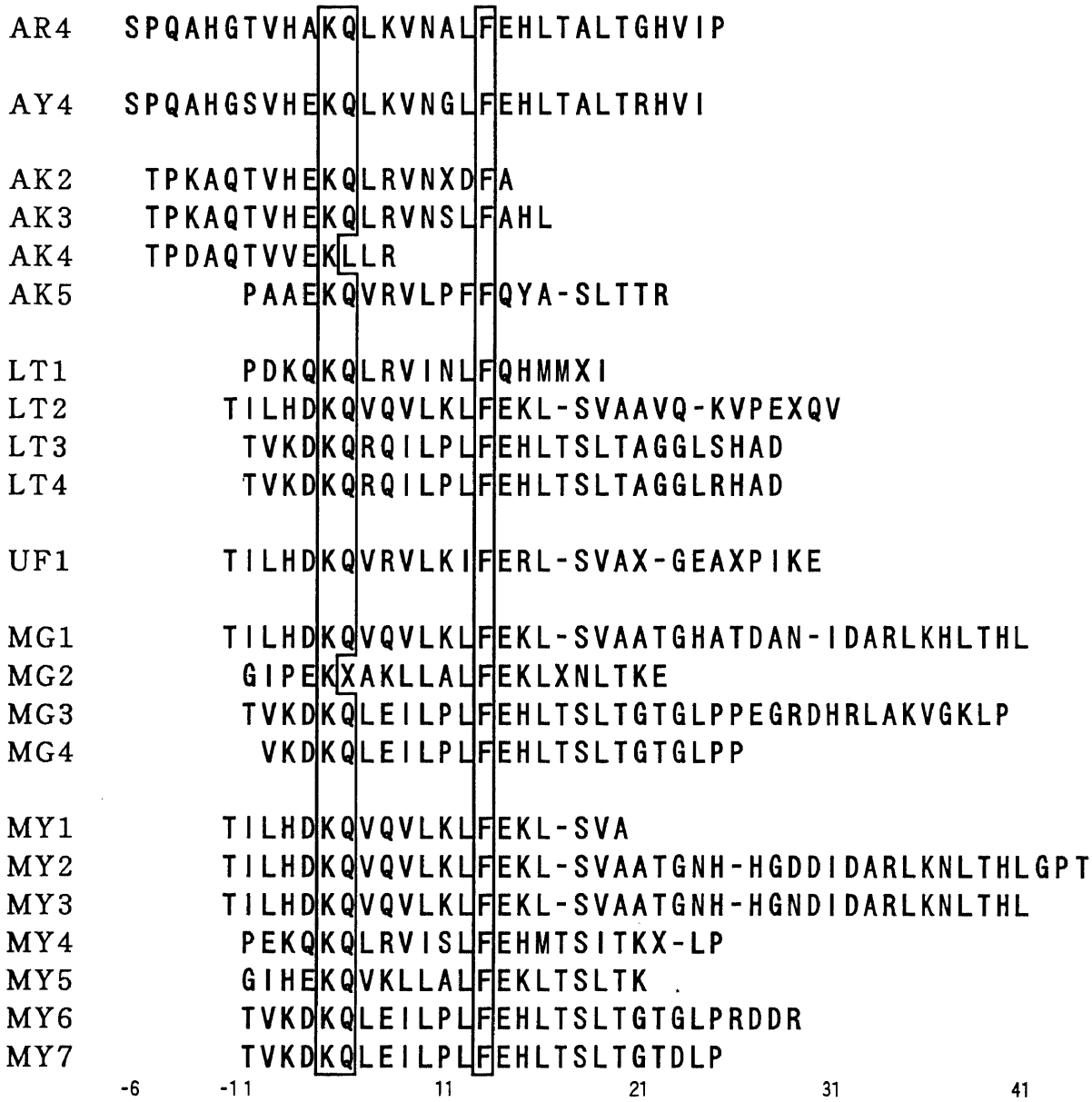

AR4 SPQAHGTVHAKQLKVNALFEHLTALTGHVIP
AY4 SPQAHGSVHEKQLKVNGLFEHLTALTRHVI
AK2 TPKAQTVHEKQLRVNXDFA
AK3 TPKAQTVHEKQLRVNSLFAHL
AK4 TPDAQTVVEKLLR
AK5 PAAEKQVRVLPFFQYA-SLTTR
LT1 PDKQKQLRVINLFQHMMXI
LT2 TILHDKQVQVLKLFEKL-SVAAVQ-KVPEXQV
LT3 TVKDKQRQILPLFEHLTSLTAGGLSHAD
LT4 TVKDKQRQILPLFEHLTSLTAGGLRHAD
UF1 TILHDKQVRVLKIFERL-SVAX-GEAXPIKE
MG1 TILHDKQVQVLKLFEKL-SVAATGHATDAN-IDARLKHLTHL
MG2 GIPEKXAKLLALFEKLXNLTKE
MG3 TVKDKQLEILPLFEHLTSLTGTGLPPEGRDHRLAKVGKLP
MG4 VKDKQLEILPLFEHLTSLTGTGLPP
MY1 TILHDKQVQVLKLFEKL-SVA
MY2 TILHDKQVQVLKLFEKL-SVAATGNH-HGDDIDARLKNLTHLGPT
MY3 TILHDKQVQVLKLFEKL-SVAATGNH-HGNDIDARLKNLTHL
MY4 PEKQKQLRVISLFEHMTSITKX-LP
MY5 GIHEKQVKLLALFEKLTSLTK
MY6 TVKDKQLEILPLFEHLTSLTGTGLPRDDR
MY7 TVKDKQLEILPLFEHLTSLTGTDLP
-6
-11
11
21
31
41

Figure 18.

N-terminal amino acid sequences of constituent monomers of hemocyanin dimer subunits from *A. roretzi* (ARd1 and d2), *A. karschi* (AKd1a and d1b), *L. typica* (LTd1a and d1b), and *M. gigas* (MGd1a, d1b, d2a and d2b). The alignment was made by hand. Amino acid residues indicated by X are ambiguous. The conservative amino acid residues in positions 5, 6 and 13 are boxed.

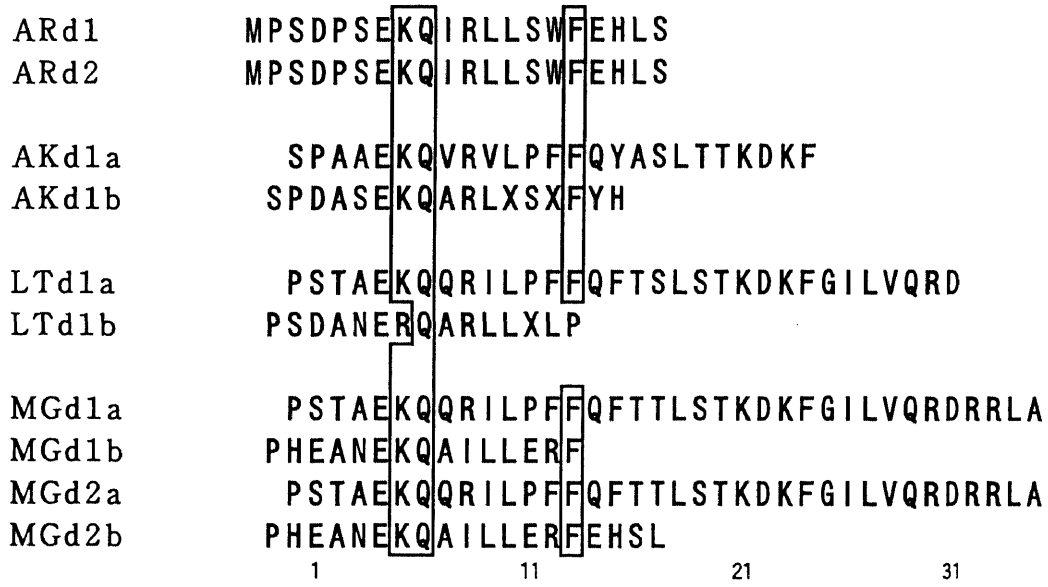

ARd1 MPSDPSEKQIRLLSWFEHLS
ARd2 MPSDPSEKQIRLLSWFEHLS
AKd1a SPAAEKQVRVLPFFQYASLTTKDKF
AKd1b SPDASEKQARLXSXFYH
LTd1a PSTAEKQQRILPFFQFTSLSTKDKFGILVQRD
LTd1b PSDANERQARLLXLP
MGd1a PSTAEKQQRILPFFQFTTLSTKDKFGILVQRDRRLA
MGd1b PHEANEKQAILLERF
MGd2a PSTAEKQQRILPFFQFTTLSTKDKFGILVQRDRRLA
MGd2b PHEANEKQAILLERFEHSL
1
11
21
31

Figure 19.

The distribution of the hemocyanin subunits in mygalomorph spiders. The monomer subunits of groups A and B, the monomer subunits of groups C, D, E and F and the constituent monomer subunits of groups G and H are shown separately in A, B and C. *A. r.*, *A. y.*, *A. k.*, *L. t.*, *U. f.*, *M. g.*, *M. y.* and *A. c.* denote *A. roretzi*, *A. yesoensis*, *A. karschi*, *L. typica*, *U. fragaria*, *M. gigas*, *M. yagimumai* and *A. californicum*, respectively. The duplications and losses of the subunits were inferred from comparison of the subunit distribution patterns. ? means unknown sequences.

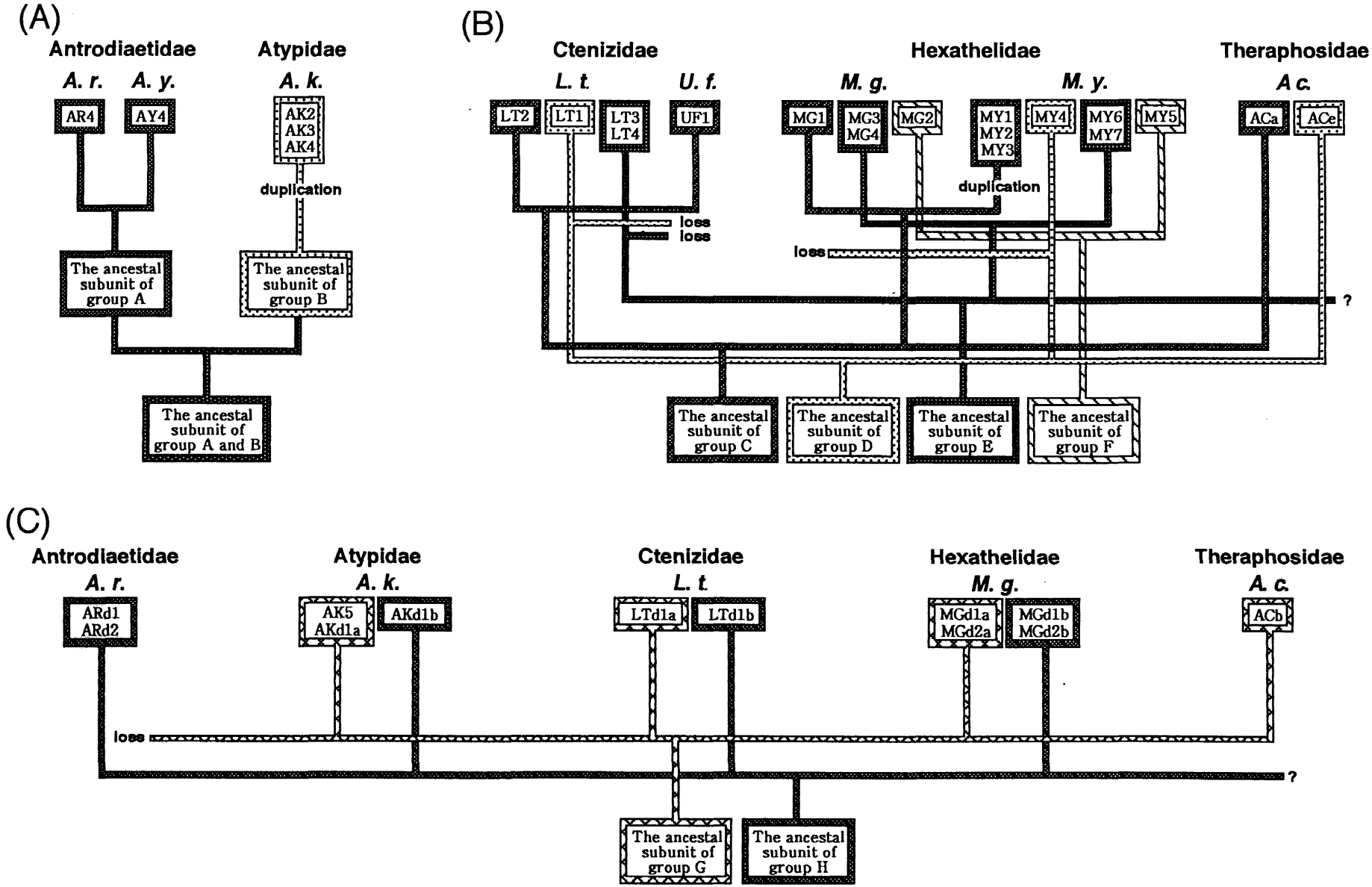

(A)
Antrodiaetidae
Atypidae
A. r.
A. y.
A. k.
AR4
AY4
AK2
AK3
AK4
duplication
The ancestal subunit of group A
The ancestal subunit of group B
The ancestal subunit of group A and B
(B)
Ctenizidae
Hexathelidae
Theraphosidae
L. t.
U. f.
M. g.
M. y.
A c.
LT2
LT1
LT3
LT4
UF1
MG1
MG3
MG4
MG2
MY1
MY2
MY3
MY4
MY6
MY7
MY5
ACa
ACe
duplication
loss
loss
loss
?
The ancestal subunit of group C
The ancestal subunit of group D
The ancestal subunit of group E
The ancestal subunit of group F
(C)
Antrodiaetidae
Atypidae
Ctenizidae
Hexathelidae
Theraphosidae
A. r.
A. k.
L. t.
M. g.
A. c.
ARd1
ARd2
AK5
AKd1a
AKd1b
LTd1a
LTd1b
MGd1a
MGd2a
MGd1b
MGd2b
ACb
loss
?
The ancestal subunit of group G
The ancestal subunit of group H

Figure 20.

Two cladograms of mygalomorph spider families proposed by Raven (A) and Goloboff (B). The family groups framed by a solid line were monophyletic in both studies. The Mygalomorphae was divided into two monophyletic groups framed by a broken line respectively by Raven (A) and Goloboff (B). Concerning the position of Rastelloidina, their classification systems of them are different.

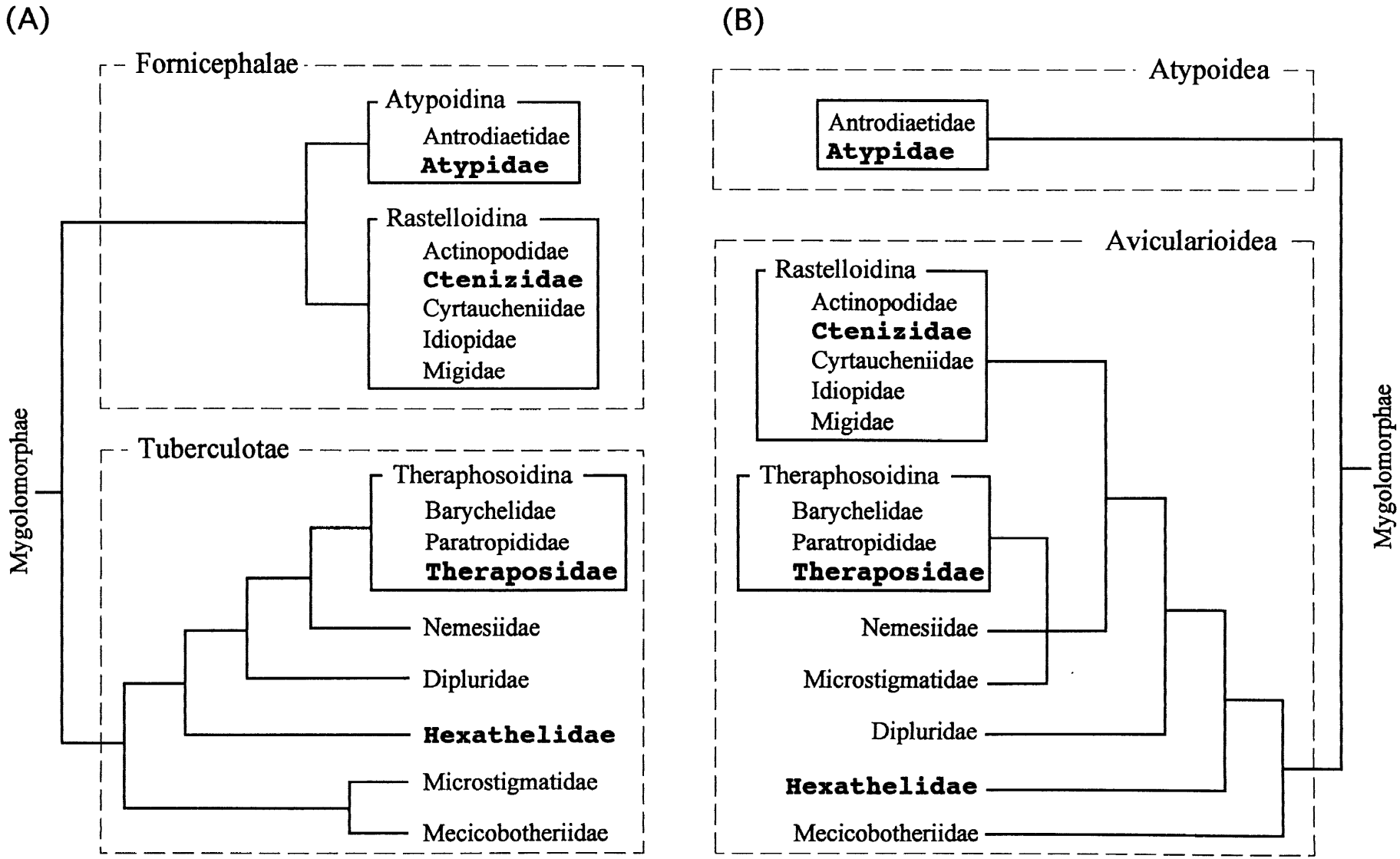
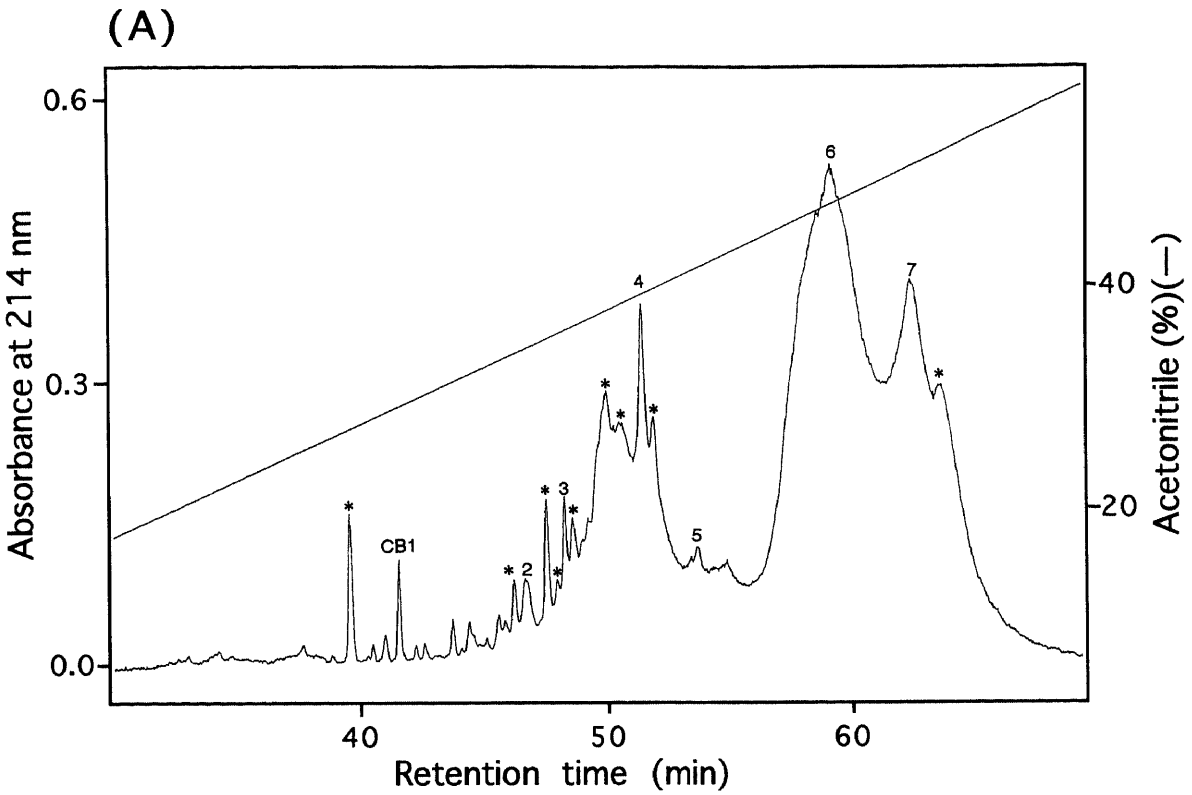

(A)
Fornicephalae
Atypoidina
Antrodiaetidae
Atypidae
Rastelloidina
Actinopodidae
Ctenizidae
Cyrtaucheniidae
Idiopidae
Migidae
Mygolomorphae
Tuberculotae
Theraphosoidina
Barychelidae
Paratropididae
Theraposidae
Nemesiidae
Dipluridae
Hexathelidae
Microstigmatidae
Mecicobotheriidae
(B)
Atypoidea
Antrodiaetidae
Atypidae
Avicularioidea
Rastelloidina
Actinopodidae
Ctenizidae
Cyrtaucheniidae
Idiopidae
Migidae
Theraphosoidina
Barychelidae
Paratropididae
Theraposidae
Nemesiidae
Microstigmatidae
Dipluridae
Hexathelidae
Mecicobotheriidae
Mygolomorphae

Figure 21.

Separation of CNBr fragments (A) and lysyl end peptidase-digested fragments (B) derived from the constituent monomer AKd1a of *A. karschi* hemocyanin dimer (AKd1) by reverse-phase chromatography using μRPC C2/C18 SC 2.1/10 (Pharmacia Biotech) in SMART system. The column was equilibrated with water containing 0.065% trifluoroacetic acid and the chromatography with a linear gradient from 0% to 60% of acetonitrile containing 0.055% trifluoroacetic was performed at flow rate of 50 μl/min. Sequencing of peptides in numbered fractions (CB1-7 and K1-14) was performed. The peptides in fractions asterisked were analyzed, but their sequences were ambiguous or same with those of adjoining fractions. Other fractions were not analyzed because it is inferred that they contain no sufficient amount of peptides for sequence analysis or peptides from enzyme self-digestion.


(A)
0.6
0.3
0.0
Absorbance at 214 nm
40
50
60
Retention time (min)
40
20
Acetonitrile (%)(—)
CB1
1
2
3
4
5
6
7

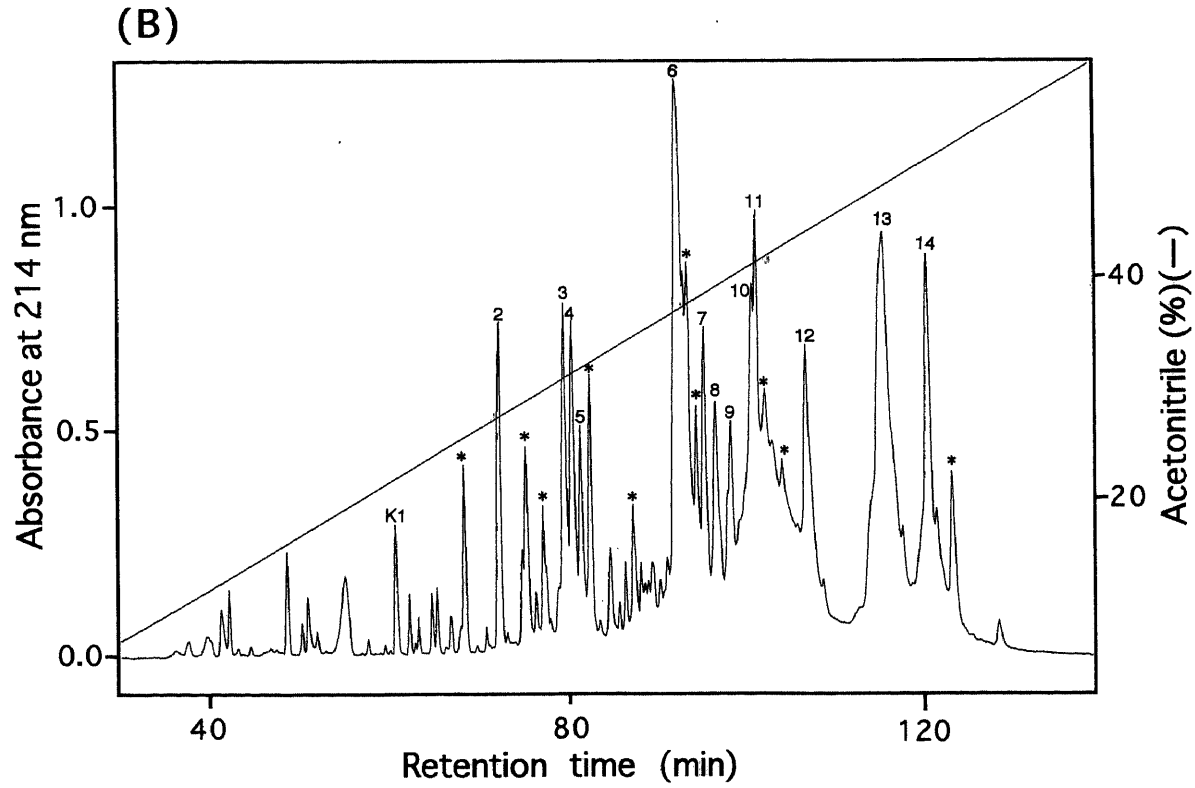

(B)
1.0
0.5
0.0
Absorbance at 214 nm
40
80
120
Retention time (min)
40
20
Acetonitrile (%)(—)
K1
1
2
3
4
5
6
7
8
9
10
11
12
13
14

Figure 22.

Comparison of aligned sequence of peptides from the constituent monomer AKd1a of *A. karschi* hemocyanin dimer (AKd1) with the amino acid sequence of *A. californicum* hemocyanin subunit ACb (from Sonner *et al.*, 1990). The alignment was made by hand. *CB*, CNBr fragments; *K*, lysyl end peptidase-digested fragments. Putative methionine and lysyl residues were underlined. Amino acid residues indicated by X are ambiguous. A dash represents a gap introduced under the necessity of comparing sequences.

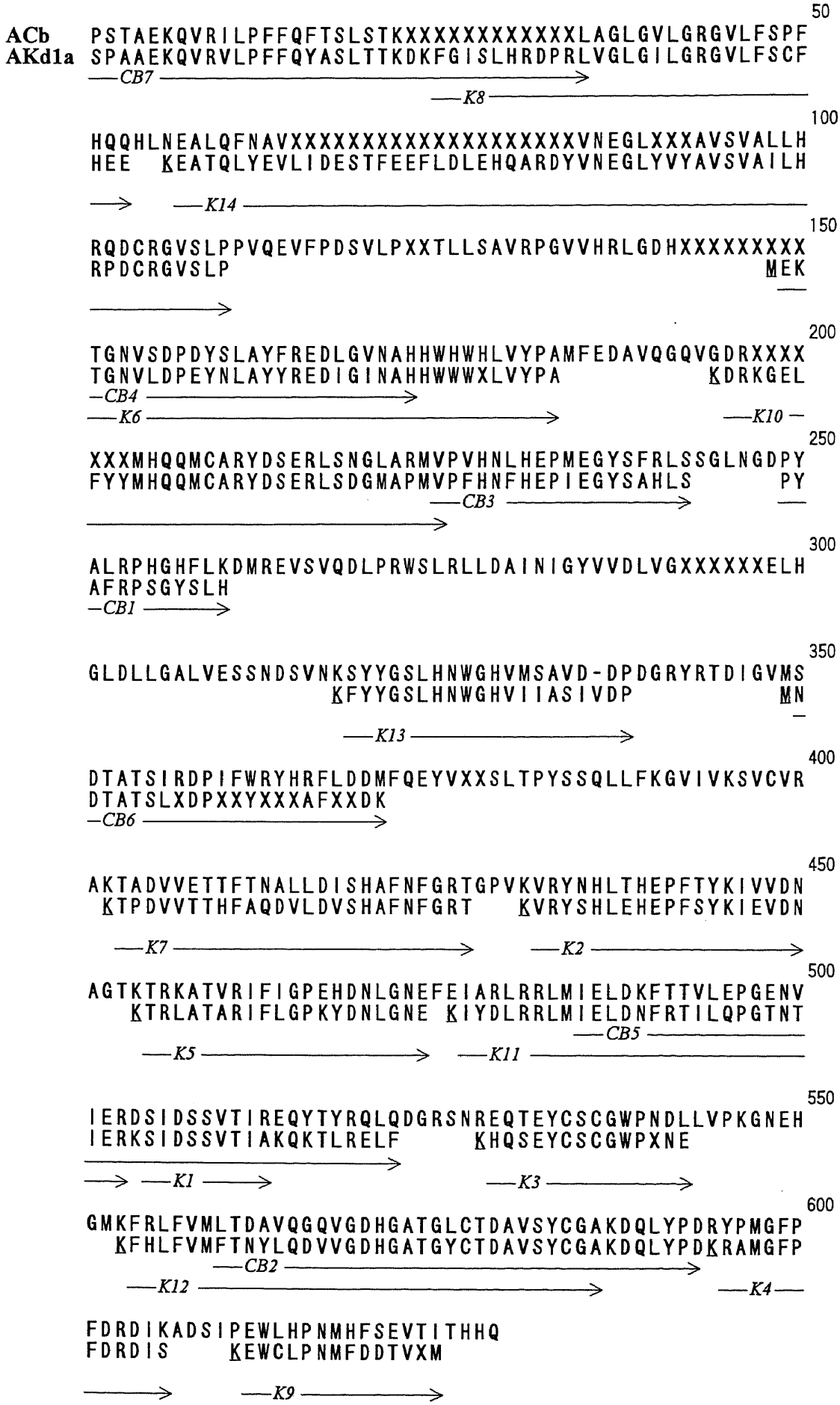
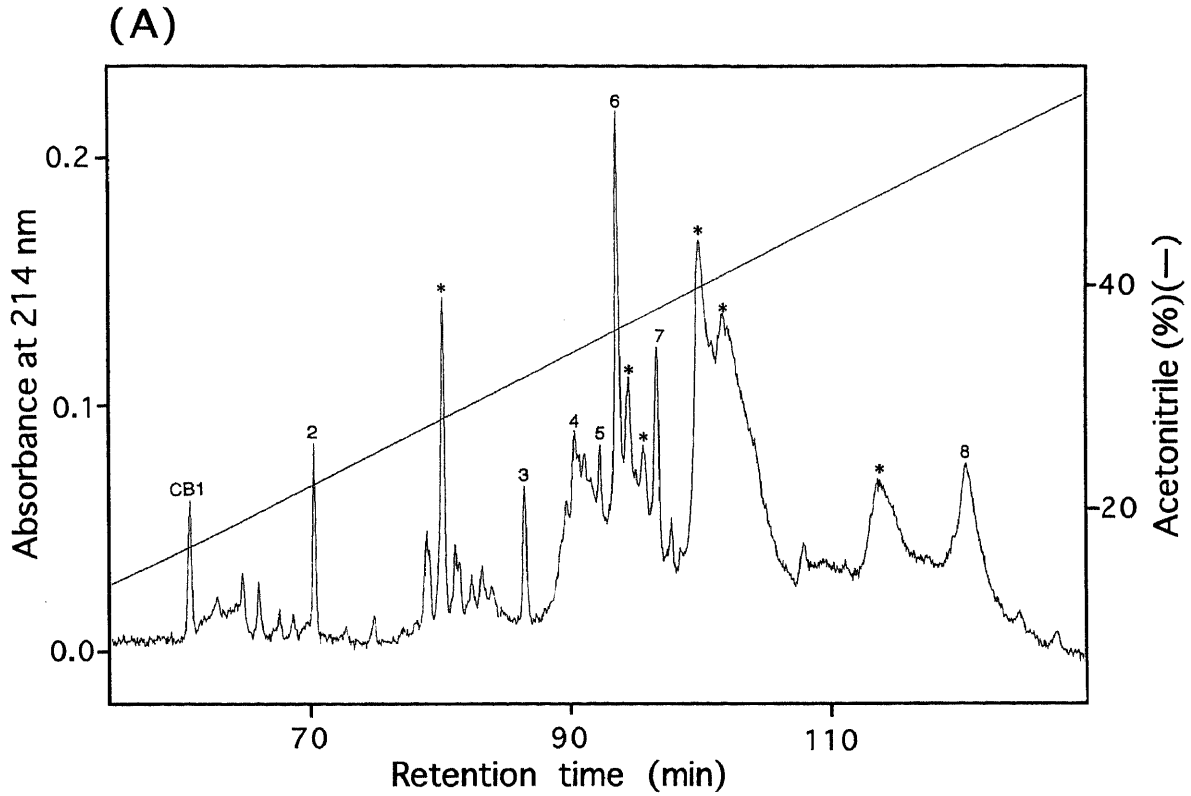

ACb
AKd1a
50
100
150
200
250
300
350
400
450
500
550
600
CB7
K8
K14
CB4
K6
K10
CB3
CB1
K13
CB6
K7
K2
CB5
K5
K11
K1
K3
CB2
K12
K4
K9

Figure 23.

Separation of CNBr fragments (A) and lysyl end peptidase-digested fragments (B) derived from the constituent monomer LTd1a of *L. typica* hemocyanin dimer (LTd1) by reverse-phase chromatography using μRPC C2/C18 SC 2.1/10 (Pharmacia Biotech)) in SMART system. Sequencing of peptides in numbered fractions (CB1-8 and K1-12) was performed. For explanations see Fig. 21.


(A)
Absorbance at 214 nm
0.2
0.1
0.0
CB1
2
3
4
5
6
7
8
*
70
90
110
Retention time (min)
40
20
Acetonitrile (%)(—)

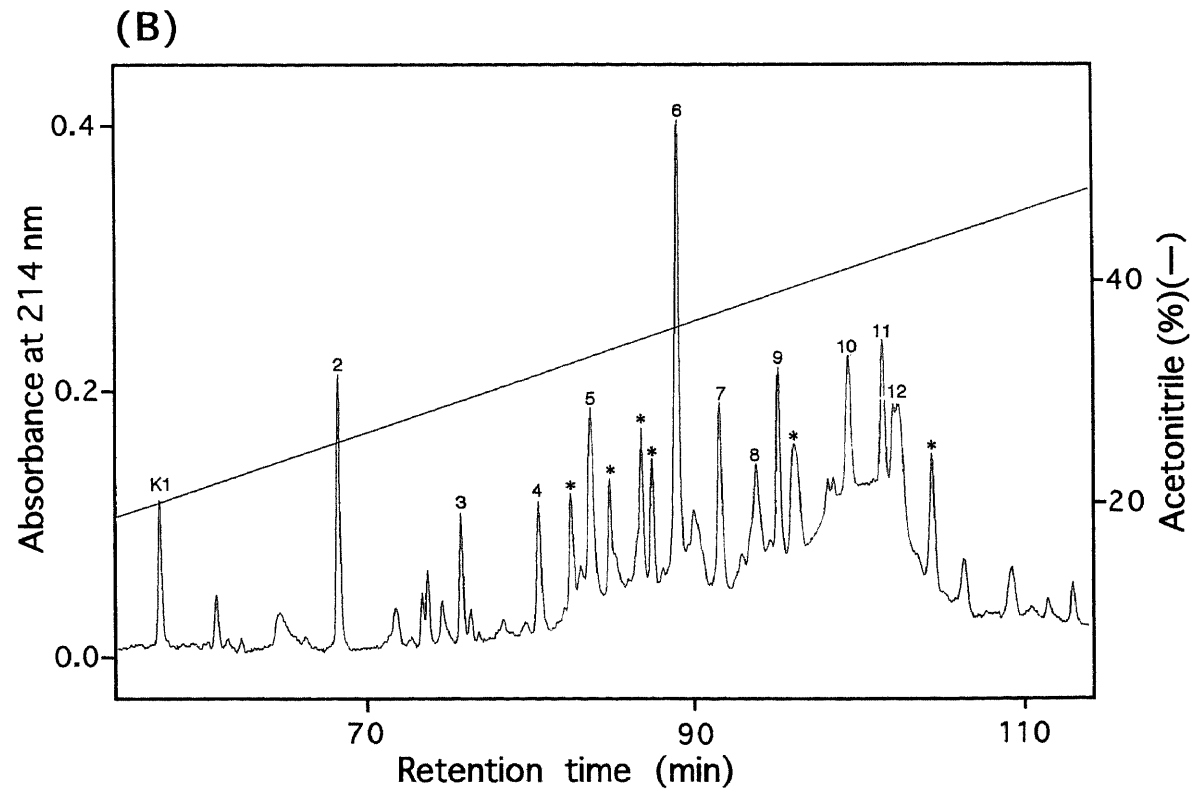

(B)
Absorbance at 214 nm
0.4
0.2
0.0
K1
2
3
4
5
6
7
8
9
10
11
12
*
70
90
110
Retention time (min)
40
20
Acetonitrile (%)(—)

Figure 24.

Comparison of aligned sequence of peptides from the constituent monomer LTd1a of *L. typica* hemocyanin dimer (LTd1) with the amino acid sequence of *A. californicum* subunit ACb (from Sonner *et al.*, 1990). For explanations see Fig. 22.

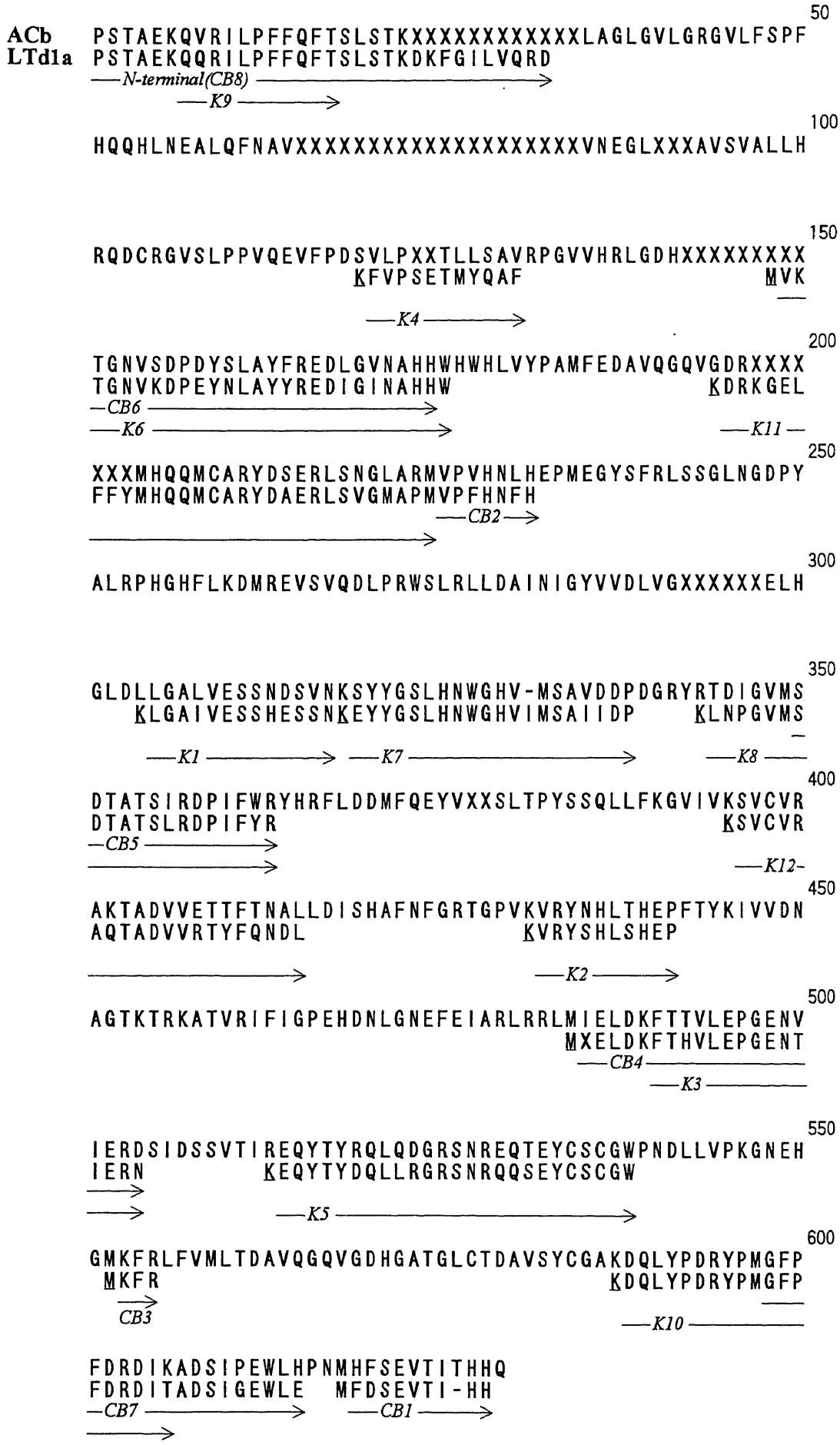
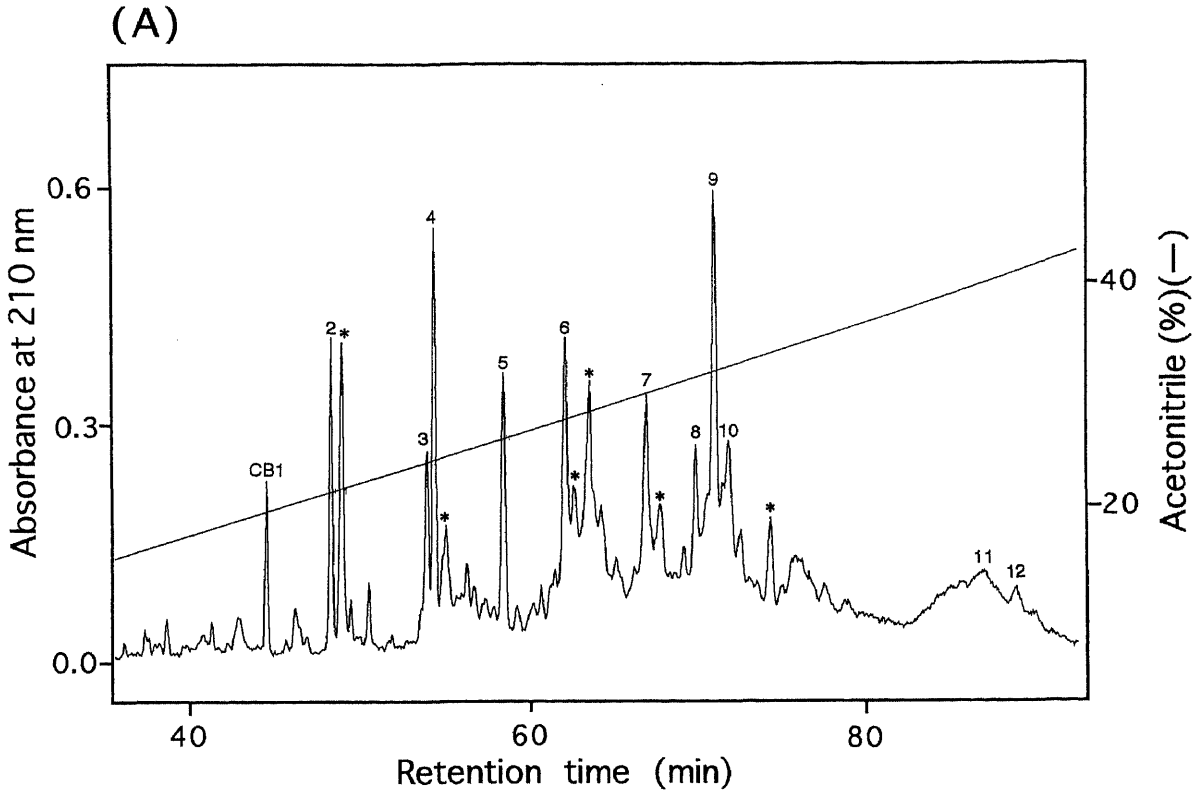

ACb PSTAEKQVRILPFFQFTSLSTKXXXXXXXXXXXXLAGLGVLGRGVLFSPF
LTd1a PSTAEKQQRILPFFQFTSLSTKDKFGILVQRD
—N-terminal(CB8)—
—K9—
HQQHLNEALQFNAVXXXXXXXXXXXXXXXXXXXXVNEGLXXXAVSVALLH
RQDCRGVSLPPVQEVFPDSVLPXXTLLSAVRPGVVHRLGDHXXXXXXXXX
KFVPSETMYQAF
MVK
—K4—
TGNVSDPDYSLAYFREDLGVNAHHWHWHLVYPAMFEDAVQGQVGDRXXXX
TGNVKDPEYNLAYYREDIGINAHHW
KDRKGEL
—CB6—
—K6—
—K11—
XXXMHQQMCARYDSERLSNGLARMVPVHNLHEPMEGYSFRLSSGLNGDPY
FFYMHQQMCARYDAERLSVGMAPMVPFHNFH
—CB2—
ALRPHGHFLKDMREVSVQDLPRWSLRLLDAINIGYVVDLVGXXXXXXELH
GLDLLGALVESSNDSVNKSYYGSLHNWGHV-MSAVDDPDGRYRTDIGVMS
KLGAIVESSHESSNKEYYGSLHNWGHVIMSAIIDP
KLNPGVMS
—K1—
—K7—
—K8—
DTATSIRDPIFWRYHRFLDDMFQEYVXXSLTPYSSQLLFKGVIVKSVCVR
DTATSLRDPIFYR
KSVCVR
—CB5—
—K12-
AKTADVVETTFTNALLDISHAFNFGRTGPVKVRYNHLTHEPFTYKIVVDN
AQTADVVRTYFQNDL
KVRYSHLSHEP
—K2—
AGTKTRKATVRIFIGPEHDNLGNEFEIARLRRLMIELDKFTTVLEPGENV
MXELDKFTHVLEPGENT
—CB4—
—K3—
IERDSIDSSVTIREQYTYRQLQDGRSNREQTEYCSCGWPNDLLVPKGNEH
IERN
KEQYTYDQLLRGRSNRQQSEYCSCGW
—K5—
GMKFRLFVMLTDAVQGQVGDHGATGLCTDAVSYCGAKDQLYPDRYPMGFP
MKFR
KDQLYPDRYPMGFP
CB3
—K10—
FDRDIKADSIPEWLHPNMHFSEVTITHHQ
FDRDITADSIGEWLE MFDSEVTI-HH
—CB7—
—CB1—

Figure 25.

Separation of CNBr fragments (A) and lysyl end peptidase-digested fragments (B) derived from the constituent monomer MGd1a of *M. gigas* hemocyanin dimer (MGd1) by reverse-phase chromatography using Sephasil C18 SC 2.1/10 (Pharmacia Biotech) in SMART system. Sequencing of peptides in numbered fractions (CB1-12 and K1-14) was performed. For explanations see Fig. 21.


(A)
Absorbance at 210 nm
0.6
0.3
0.0
CB1
2
3
4
5
6
7
8
9
10
11
12
40
60
80
Retention time (min)
40
20
Acetonitrile (%)(—)

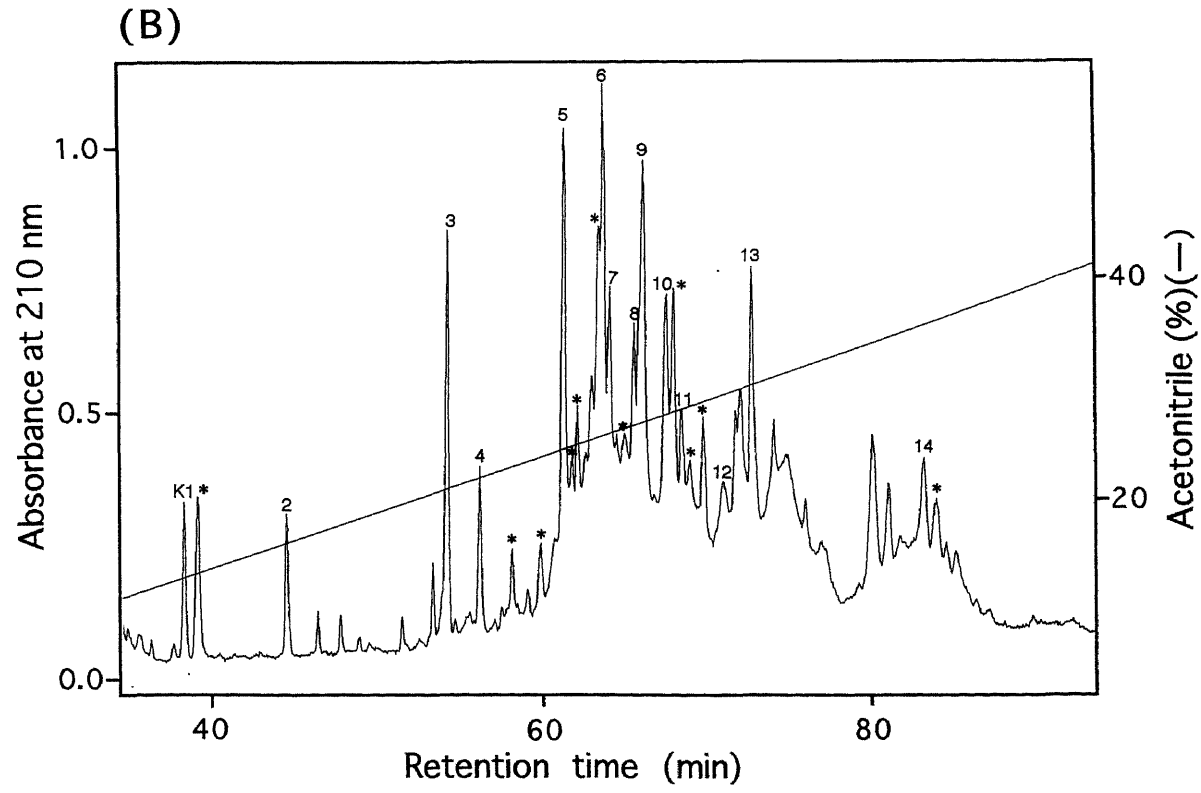

(B)
Absorbance at 210 nm
1.0
0.5
0.0
K1
2
3
4
5
6
7
8
9
10
11
12
13
14
40
60
80
Retention time (min)
40
20
Acetonitrile (%)(—)

Figure 26.

Comparison of aligned sequence of peptides from the constituent monomer MGd1a of *M. gigas* hemocyanin dimer (MGd1) with the amino acid sequence of *A. californicum* subunit ACb (from Sonner *et al.*, 1990). CB11-V1 was a fragment derived from a CNBr fragment (CB11) digested with V8 protease. Putative glutamic acid residues were underlined. For explanations see Fig. 22.

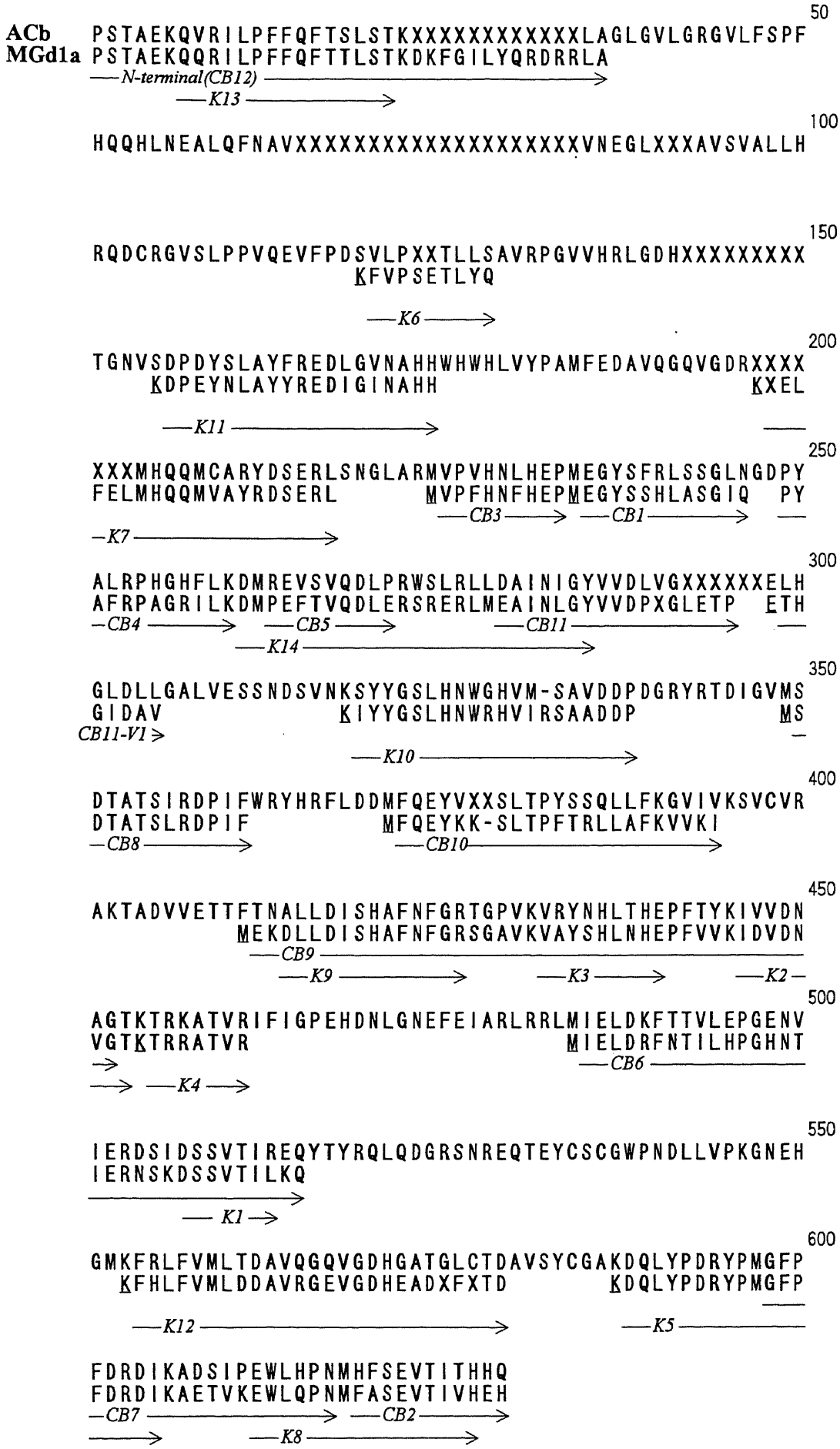
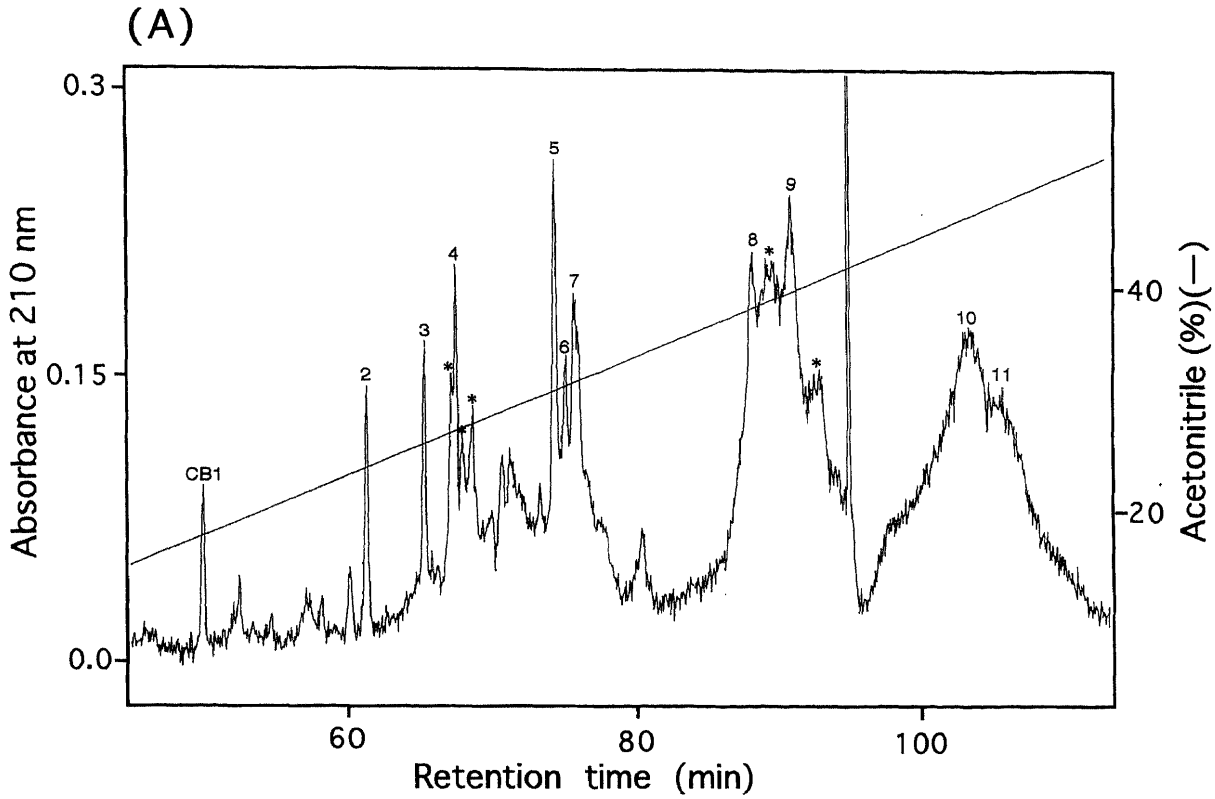

ACb
MGd1a
50
PSTAEKQVRILPFFQFTSLSTKXXXXXXXXXXXXLAGLGVLGRGVLFSPF
PSTAEKQQRILPFFQFTTLSTKDKFGILYQRDRRLA
—N-terminal(CB12)
—K13
100
HQQHLNEALQFNAVXXXXXXXXXXXXXXXXXXXXVNEGLXXXAVSVALLH
150
RQDCRGVSLPPVQEVFPDSVLPXXTLLSAVRPGVVHRLGDHXXXXXXXXX
KFVPSETLYQ
—K6
200
TGNVSDPDYSLAYFREDLGVNAHHWHWHLVYPAMFEDAVQGQVGDRXXXX
KDPEYNLAYYREDIGINAHH
KXEL
—K11
250
XXXMHQQMCARYDSERLSNGLARMVPVHNLHEPMEGYSFRLSSGLNGDPY
FELMHQQMVAYRDSERL
MVPFHNFHEPMEGYSSHLASGIQ
PY
—CB3
—CB1
—K7
300
ALRPHGHFLKDMREVSVQDLPRWSLRLLDAINIGYVVDLVGXXXXXXELH
AFRPAGRILKDMPEFTVQDLERSRERLMEAINLGYVVDPXGLETP
ETH
—CB4
—CB5
—CB11
—K14
350
GLDLLGALVESSNDSVNKSYYGSLHNWGHVM-SAVDDPDGRYRTDIGVMS
GIDAV
KIYYGSLHNWRHVIRSAADDP
MS
CB11-V1
—K10
400
DTATSIRDPIFWRYHRFLDDMFQEYVXXSLTPYSSQLLFKGVIVKSVCVR
DTATSLRDPIF
MFQEYKK-SLTPFTRLLAFKVVKI
—CB8
—CB10
450
AKTADVVETTFTNALLDISHAFNFGRTGPVKVRYNHLTHEPFTYKIVVDN
MEKDLLDISHAFNFGRSGAVKVAYSHLNHEPFVVKIDVDN
—CB9
—K9
—K3
—K2
500
AGTKTRKATVRIFIGPEHDNLGNEFEIARLRRLMIELDKFTTVLEPGENV
VGTKTRRATVR
MIELDRFNTILHPGHNT
—CB6
—K4
550
IERDSIDSSVTIREQYTYRQLQDGRSNREQTEYCSCGWPNDLLVPKGNEH
IERNSKDSSVTILKQ
—K1
600
GMKFRLFVMLTDAVQGQVGDHGATGLCTDAVSYCGAKDQLYPDRYPMGFP
KFHLFVMLDDAVRGEVGDHEADXFXTD
KDQLYPDRYPMGFP
—K12
—K5
FDRDIKADSIPEWLHPNMHFSEVTITHHQ
FDRDIKAETVKEWLQPNMFASEVTIVHEH
—CB7
—CB2
—K8

Figure 27.

Separation of CNBr fragments (A) and lysyl end peptidase-digested fragments (B) derived from the constituent monomer MGd1b of *M. gigas* hemocyanin dimer (MGd1) by reverse-phase chromatography using Sephasil C18 SC 2.1/10 (Pharmacia Biotech) in SMART system. Sequencing of peptides in numbered fractions (CB1-11 and K1-10) was performed. For explanations see Fig. 21.


(A)
0.3
0.15
0.0
Absorbance at 210 nm
CB1
2
3
4
5
6
7
8
9
10
11
40
20
Acetonitrile (%)(—)
60
80
100
Retention time (min)

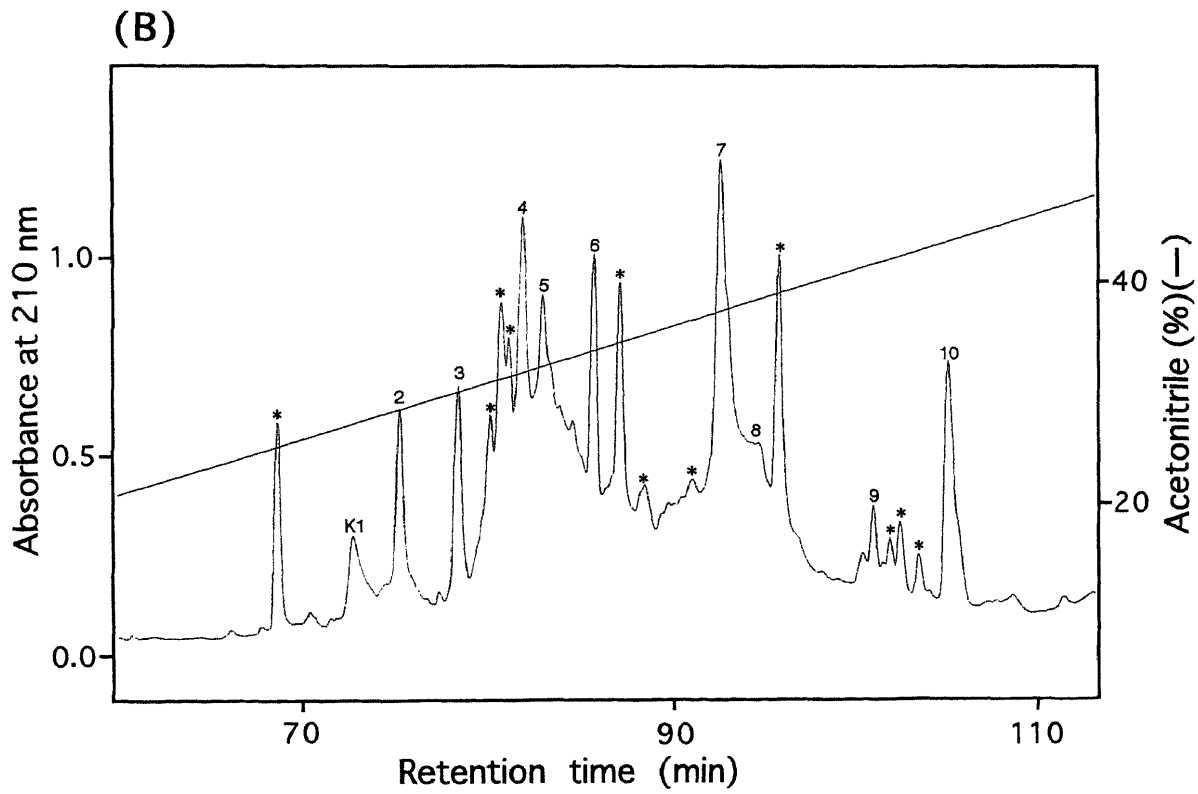

(B)
1.0
0.5
0.0
Absorbance at 210 nm
K1
2
3
4
5
6
7
8
9
10
40
20
Acetonitrile (%)(—)
70
90
110
Retention time (min)

Figure 28.

Comparison of aligned sequence of peptides from the constituent monomer MGd1a of *M. gigas* hemocyanin dimer (MGd1) with the amino acid sequence of *A. californicum* subunit ACb (from Sonner *et al.*, 1990). CB8-V1 and V2 are the peptides derived from a CNBr fragment (CB8) digested with V8 protease. Putative glutamic acid residues were underlined. For explanations see Fig. 22.

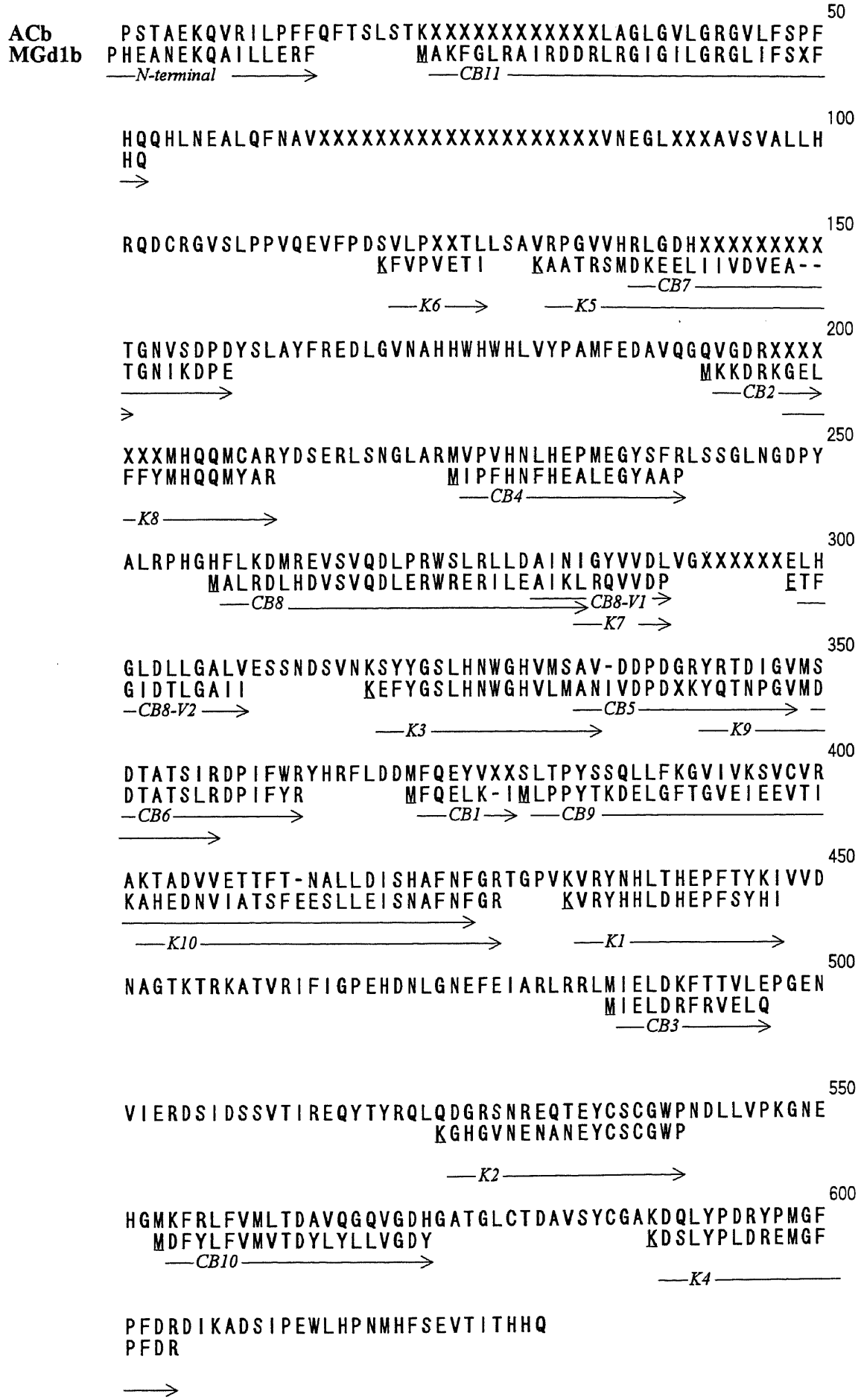
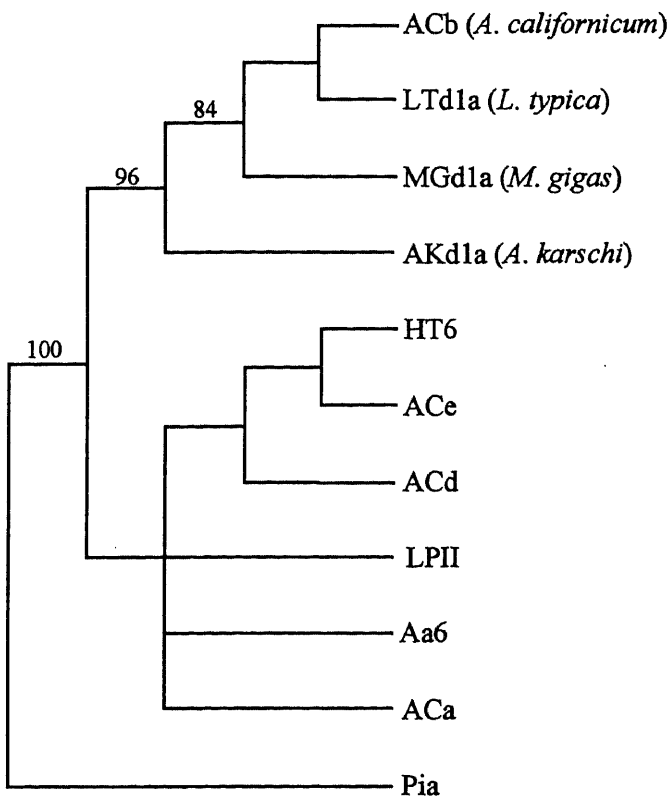

ACb
MGd1b
50
PSTAEKQVRILPFFQFTSLSTKXXXXXXXXXXXXLAGLGVLGRGVLFSPF
PHEANEKQAILLERF MAKFGLRAIRDDRLRGIGILGRGLIFSXF
—N-terminal
—CB11
100
HQQHLNEALQFNAVXXXXXXXXXXXXXXXXXXXXVNEGLXXXAVSVALLH
HQ
150
RQDCRGVSLPPVQEVFPDSVLPXXTLLSAVRPGVVHRLGDHXXXXXXXXX
KFVPVETI KAATRSMDKEELIIVDVEA--
—CB7
—K6
—K5
200
TGNVSDPDYSLAYFREDLGVNAHHWHWHLVYPAMFEDAVQGQVGDRXXXX
TGNIKDPE MKKDRKGEL
—CB2
250
XXXMHQQMCARYDSERLSNGLARMVPVHNLHEPMEGYSFRLSSGLNGDPY
FFYMHQQMYAR MIPFHNFHEALEGYAAP
—CB4
—K8
300
ALRPHGHFLKDMREVSVQDLPRWSLRLLDAINIGYVVDLVGXXXXXXELH
MALRDLHDVSVQDLERWRERILEAIKLRQVVDP ETF
—CB8
CB8-V1
—K7
350
GLDLLGALVESSNDSVNKSYYGSLHNWGHVMSAV-DDPDGRYRTDIGVMS
GIDTLGAII KEFYGSLHNWGHVLMANIVDPDXKYQTNPGVMD
—CB8-V2
—CB5
—K3
—K9
400
DTATSIRDPIFWRYHRFLDDMFQEYVXXSLTPYSSQLLFKGVIVKSVCVR
DTATSLRDPIFYR MFQELK-IMLPPYTKDELGFTGVEIEEVTI
—CB6
—CB1
—CB9
450
AKTADVVETTFT-NALLDISHAFNFGRTGPVKVRYNHLTHEPFTYKIVVD
KAHEDNVIATSFEESLLEISNAFNFGR KVRYHHLDHEPFSYHI
—K10
—K1
500
NAGTKTRKATVRIFIGPEHDNLGNEFEIARLRRLMIELDKFTTVLEPGEN
MIELDRFRVELQ
—CB3
550
VIERDSIDSSVTIREQYTYRQLQDGRSNREQTEYCSCGWPNDLLVPKGNE
KGHGVNENANEYCSCGWP
—K2
600
HGMKFRLFVMLTDAVQGQVGDHGATGLCTDAVSYCGAKDQLYPDRYPMGF
MDFYLFVMVTDYLYLLVGDY KDSLYPLDREMGF
—CB10
—K4
PFDRDIKADSIPEWLHPNMHFSEVTITHHQ
PFDR

Figure 29.

The evolutionary tree of hemocyanin subunits including the constituent monomers of group G. (A) Strict consensus tree of two most parsimonious (MP) trees. Tree length is 623 steps. (B) Neighbor joining (NJ) tree. Scale bar represents Dayhoff PAM matrix distance. Amino acids in 171 sites of the aligned sequences of hemocyanin subunits were used for phylogenetic analyses. The bootstrap values greater than 80% are shown above branches. AKd1a, subunit of *A. karschi*; LTd1a, subunit of *L. typica*; MGd1a, subunit of *M. gigas*; ACb, subunit of *A. californicum* (Sonner, *et al.* 1990); ACa (Voit and Feldmaier-Fuchs, 1990), d (Schartau *et al.*, 1983) and e (Schneider *et al.*, 1983), *A. californicum* hemocyanin monomers; HT6, hemocyanin monomer of Japanese horseshoe crab, *T. tridentatus* (Linzen *et al*, 1985); Lp II, hemocyanin monomer of American horseshoe crab, *L. polyphemus* (Nakashima *et al.*, 1986); Aa6, hemocyanin monomer of a scorpion, *Androctonus australis* (Buzy *et al.*, 1995); Pia, hemocyanin monomer of the spiny lobster, *Panulirus interruptus* (Bak and Beintema, 1987).

(A)


ACb (A. californicum)
LTd1a (L. typica)
MGd1a (M. gigas)
AKd1a (A. karschi)
HT6
ACe
ACd
LPII
Aa6
ACa
Pia
84
96
100

(B)

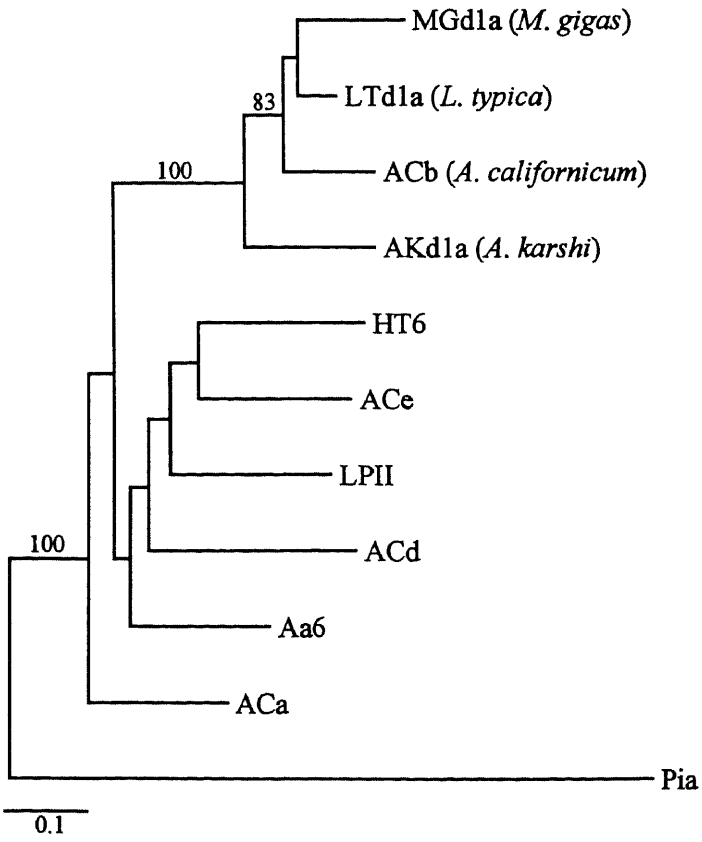
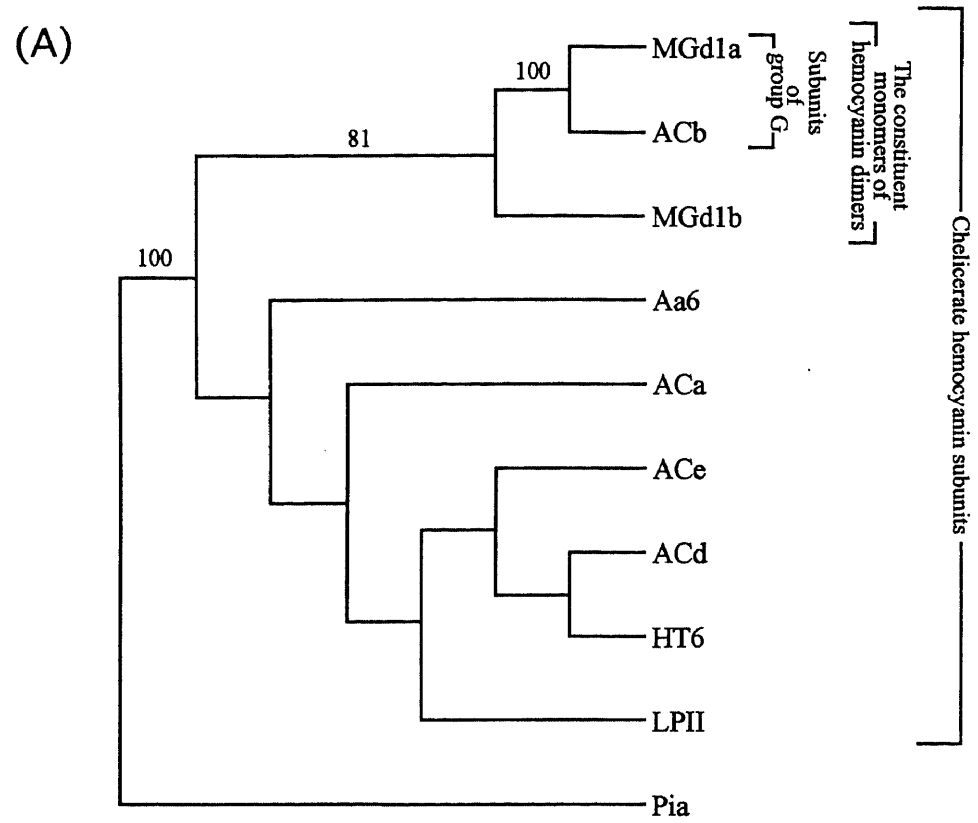

MGd1a (M. gigas)
LTd1a (L. typica)
ACb (A. californicum)
AKd1a (A. karshi)
HT6
ACe
LPII
ACd
Aa6
ACa
Pia
0.1
83
100
100

Figure 30.

The evolutionary tree of hemocyanin subunits including the constituent monomers of group G (MGd1a and ACb) and H (MGd1b). (A) The most parsimonious (MP) tree. Tree length is 893 steps. (B) Neighbor joining (NJ) tree. Scale bar represents Dayhoff PAM matrix distance. Amino acids in 233 sites of the aligned sequences of hemocyanin subunits were used for phylogenetic analyses. For explanations see Fig. 29.


(A)
MGd1a
ACb
MGd1b
Aa6
ACa
ACe
ACd
HT6
LPII
Pia
100
81
100
Subunits of group G
The constituent monomers of hemocyanin dimers
Chelicerate hemocyanin subunits

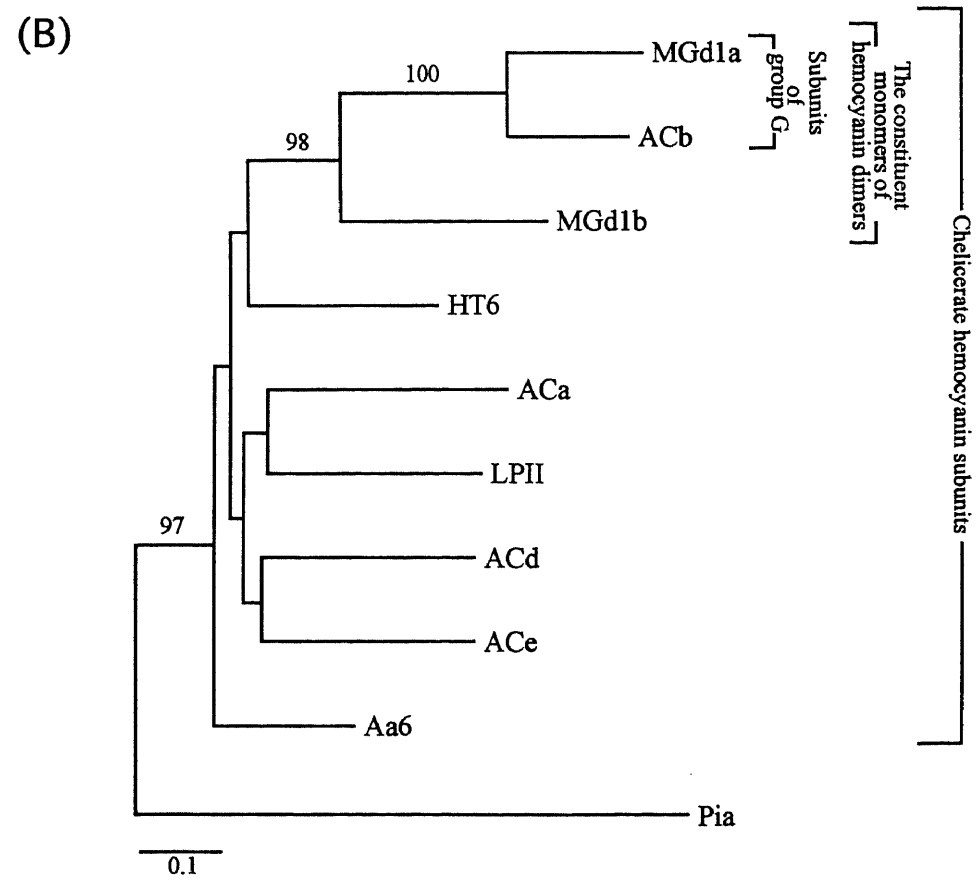
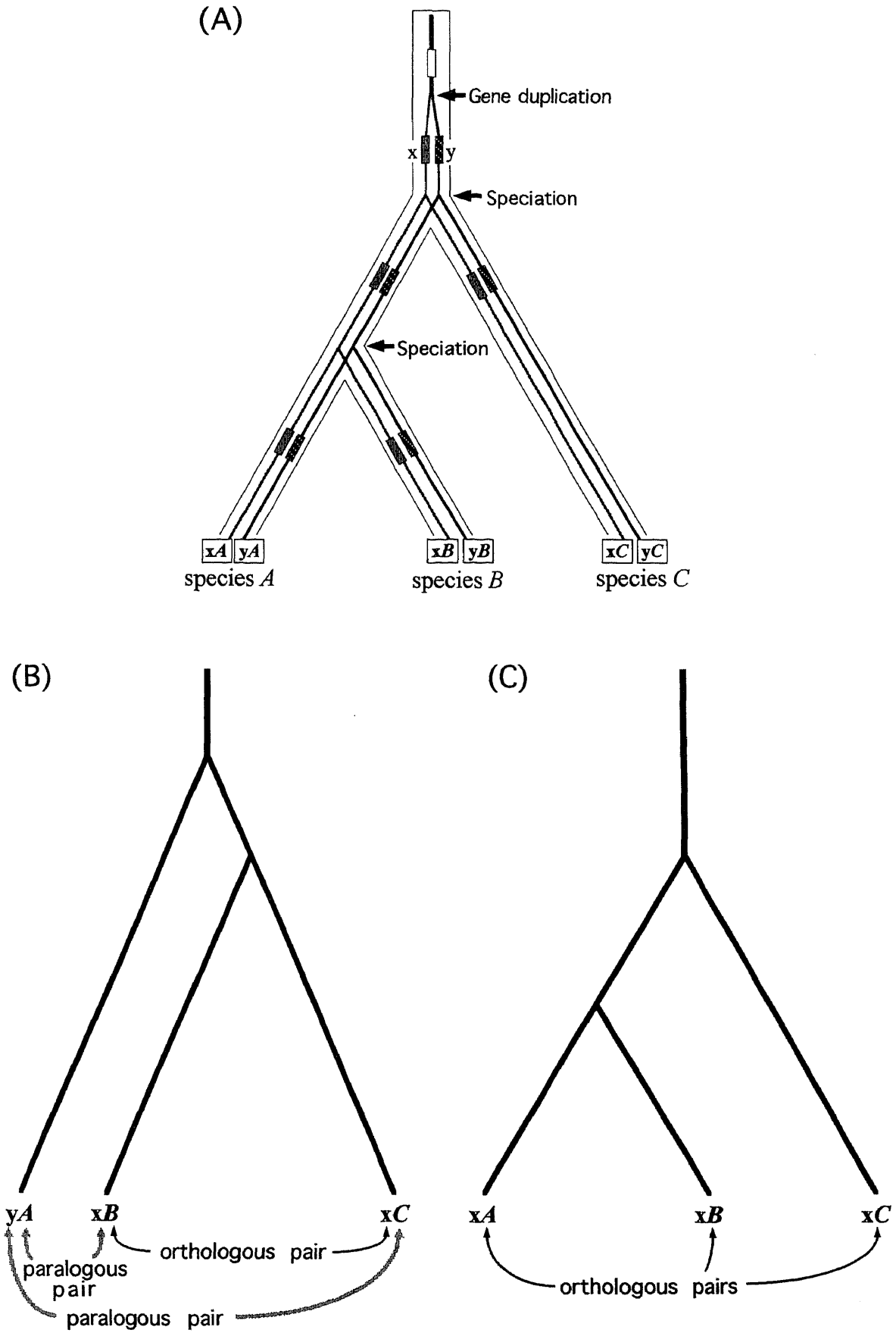

(B)
MGd1a
ACb
MGd1b
HT6
ACa
LPII
ACd
ACe
Aa6
Pia
100
98
97
0.1
Subunits of group G
The constituent monomers of hemocyanin dimers
Chelicerate hemocyanin subunits

Figure 31.

The species tree and the gene tree. The gene duplication occurred in a common ancestral lineage of three species A, B and C and yielded a paralogous pair of genes (x and y), and the two genes are shared with descendent species through speciation (A). If the paralogous pairs, such as yA and xB, and yA and xC, are used for phylogenetic study, the tree is not congruent with the evolutionary history of animal species (B). On the other hand, three genes xA, xB and xC, or genes yA, yB and yC are orthologous, because they are derived from a common ancestral gene x or y, respectively. The divergence pattern of three orthologous genes is congruent with divergence pattern of three species (C).


(A)
Gene duplication
x
y
Speciation
Speciation
xA
yA
xB
yB
xC
yC
species A
species B
species C
(B)
yA
xB
xC
orthologous pair
paralogous pair
paralogous pair
(C)
xA
xB
xC
orthologous pairs