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Subunit composition and N-terminal analysis of arthropod hemocyanins

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Abstract

Fourteen structural subunits of hemocyanins from two different infraorders of crustacea, brachyura (*Maia squinado*, *Carcinus maenas*) and astacidea (*Homarus americanus*) were isolated and characterized. N-terminal amino acid sequences were determined and compared with known sequences of crustacean and cheliceratan hemocyanins. Relationships between the investigated polypeptide chains were established. The results demonstrate that the degree of identity, calculated from the amino terminal sequences, is lower than that determined from the complete sequences and can be used for reliable characterization of the whole individual subunits. Independent evolution is possible not only for members of different subphyla, but also for those from one infraorder or from the same hemocyanin.

Keywords: Arthropod; Chelicerate; Crustacea; Hemocyanin; Sequence similarity; N-terminal sequence; Subunit; Evolution

1. Introduction

Hemocyanins are a family of copper proteins which carry dioxygen to the tissues of invertebrates from the two phyla: arthropods and molluscs. They are freely dissolved in the hemolymph in concentrations up to 120 mg/ml. The first hemocyanin had been isolated in 1878 from the hemolymph of *Octopus vulgaris* [7]. After a long period of 'break', these respiratory proteins have been studied intensively worldwide during the last decades. It was shown that arthropod hemocyanins are hexamers or multi-hexameric assemblies with a molecular mass of 0.45×10^6 to 3.9×10^6 daltons [9]. The aggregates are composed of subunits with an M_r of 67–90 kDa. Each subunit represents a single polypeptide chain build up of more than 600 amino acid residues and contains a single dioxygen-binding site [9]. The X-ray structures of two respiratory proteins from the arthropods *Palinurus interruptus* and *Limulus polyphemus* [25,8] have been determined at 0.320 and 0.218 nm, respectively. The molecular architecture of arthropod hemocyanins is entirely different from that of their molluscan counterparts (for reviews see [14,23,24]). Both groups of dioxygen-binding copper proteins have evolved independently from tyrosinases [2,3,13].

The aggregation level and subunit composition of hemocyanins from chelicerates and crustaceans have been analyzed by a variety of techniques [13]. As a result of these studies eight immunologically discernible subunit types have been classified. It was also demonstrated that the total set of subunits is required to reorganize the original aggregate from the polypeptide mixtures [22].

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Complete amino acid sequences of hemocyanins from several arthropodan species have been determined: *Panulirus interruptus*, subunits a [1], b [10] and c [17]; *Palinurus vulgaris* [11]; *Eurypelma californicum*, subunits d [19] and e [20]; *Limulus polyphemus* subunit II [26]; *Tachypleus tridentatus*, subunit α [15], *Androctonus australis* [4] and *Cancer magister* [5]. The sequence similarity between these respiratory proteins was assessed by comparison of the respective sequences. However, the primary structure of only three representatives of hemocyanins from the subphylum crustacea, namely *P. interruptus*, *P. vulgaris* and *C. magister* is known. This makes the formulation of general conclusions difficult to be valid for the members of the subphylum and different infraorders. Neuteboom et al. [16] started investigations on the N-terminal amino acid sequences of crustacean hemocyanin polypeptide chains and showed that this analysis discriminates between different subunit types within infraorders.

In order to study further the relationships between functional subunits of respiratory proteins from the two subphyla of arthropoda, crustacea and chelicerate, we have isolated 14 individual polypeptide chains of hemocyanins from two different infraorders of crustacea: brachyura (*Maia squinado*, *Carcinus maenas*) and astacidea (*Homarus americanus*). The N-terminal sequences of the purified subunits were determined and compared with those published till now.

2. Materials and methods

Hemocyanins from *Homarus americanus*, *Maia squinado*, *Palinurus vulgaris* and *Carcinus maenas* were purified by the method described in [18].

For dissociation of the aggregates, hemocyanins were dialyzed versus 50 mM bicarbonate buffer, pH 10.0, containing 2 M urea and 10 mM EDTA for 24 h. The mixture was chromatographed on a 37×3.0 cm column of DEAE-Sepharose CL-6B (Pharmacia, Uppsala, Sweden), equilibrated and eluted with the same buffer. Samples were desalted by gel-filtration through a column of Sephadex G-25 (Pharmacia). Individual subunits were isolated by ion-exchange FPLC on a Mono-Q column 10/10 (Pharmacia) using 50 mM bicarbonate buffer, containing 10 mM EDTA, pH 10.0 and a gradient of 0.0–1.0 M NaCl. The subunits were further purified by HPLC on a Nucleosil $7C_{18}$ column (250×10 mm; Machery–Nagel, Düren, Germany). The following conditions for the HPL chromatography were used: eluent A, 0.1% trifluoroacetic acid; eluent B, 80% acetonitrile in A; gradient program, 15% B for 5 min and after that 15–100% B in 55 min at a flow rate of 1 ml/min.

Amino acid compositions of the protein samples were determined after hydrolysis in 5.7 M HCl or in 5% thioglycolic acid in evacuated sealed tubes for 24, 48 and 72 h at 110°C. A BIOTRONIK model LC 3000 automatic amino acid analyzer was used. The values for the hemocyanin subunits are expressed as a percentage of a particular amino acid of a total. Cysteine was determined by the method of Ellman [6] after reduction of the samples with dithiothreitol under a nitrogen atmosphere.

Automated Edman degradation was performed using an Applied Biosystems pulsed liquid sequencer model 473 A (Weiterstadt, Germany) with on-line analysis of the phenylthiohydantoin derivatives. Approximately 50–200 pmol of proteins were applied on the cartridge filter previously treated with polybrene.

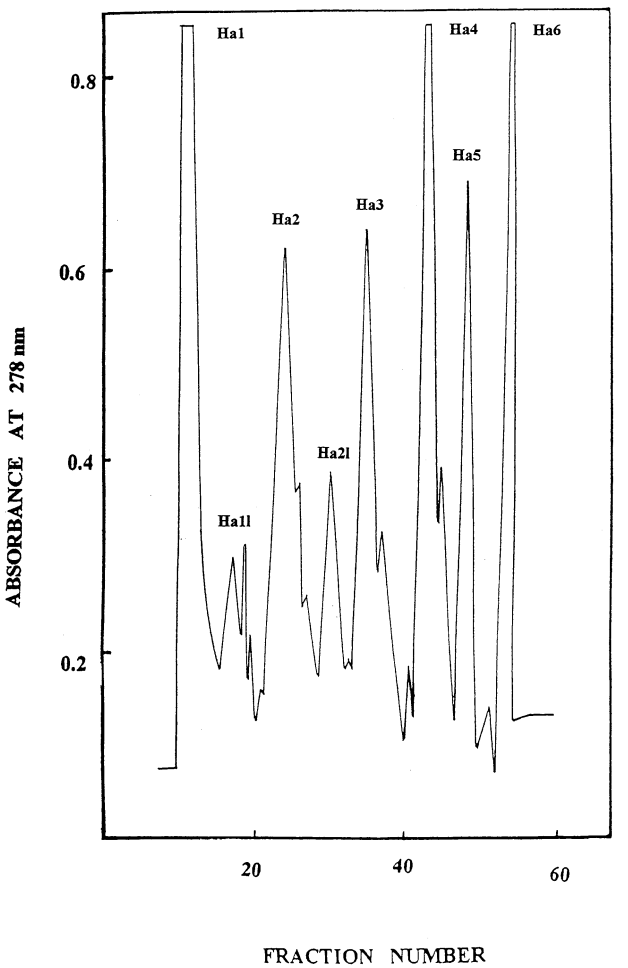

Fig. 1. Fractionation of dissociated *Homarus americanus* hemocyanin by ion-exchange FPLC on a Mono-Q column 10/10. 50 mM bicarbonate buffer, containing 10 mM EDTA, pH 10.0 and a gradient of 0.0–1.0 M NaCl were used. Six major fractions (Ha1–Ha6) were isolated.

Table 1
Amino acid compositions of the major *Homarus americanus* hemocyanin subunits

Amino acid	Ha1	Ha2	Ha3	Ha4	Ha5	Ha6
Asx	12.55	13.50	12.16	13.50	13.47	13.49
Thr	4.92	4.70	5.05	5.45	4.75	5.30
Ser	5.16	5.10	5.19	4.60	5.16	5.39
Glu	12.20	10.62	12.22	10.62	10.67	10.46
Pro	4.69	4.51	4.86	4.93	4.76	4.66
Gly	7.48	7.43	7.38	7.21	8.08	7.60
Ala	5.67	5.30	4.92	5.32	4.96	5.45
Cys	0.87	0.94	1.02	0.88	0.85	0.97
Val	5.33	5.28	5.13	5.40	4.86	4.66
Met	2.53	2.62	2.42	2.47	2.43	2.45
Ile	5.21	5.81	5.45	5.69	5.23	5.95
Leu	7.33	7.41	7.63	7.49	7.69	6.83
Tyr	4.23	4.46	4.60	4.30	4.53	4.06
Phe	5.44	5.49	5.34	5.66	5.50	5.77
His	5.45	5.30	5.71	5.69	5.83	5.69
Lys	4.77	4.99	4.81	4.81	4.60	5.19
Arg	5.04	4.84	4.67	4.80	5.28	4.54
Trp	1.17	1.28	1.20	1.19	1.35	1.39

Values are expressed as a percentage of a individual amino acid of total.
Residues were calculated from 5.7 N HCl hydrolysates at 110°C for 24, 48 and 72 h. Aspartic and glutamic acid values are the sum of their acids and amides. Tryptophan was determined in the presence of 6% thioglycolic acid. Cysteine determination was performed after reduction with dithiothreitol by the method of Ellman [6].

3. Results and discussion

Fig. 1 shows fractionation by ion-exchange FPLC of the dissociated *Homarus americanus* hemocyanin on a column Mono Q 10/10. Six major (Ha1–Ha6) and two minor (Ha1l and Ha2l) fractions were collected and purified by HPLC on a Nucleosil $7C_{18}$ column. Similar results were obtained with the other hemocyanins. In total, 14 crustacean hemocyanin subunits were isolated, rechromatographed and characterized. They were homogeneous, electrophoretically and in sequence analysis. The electrophoretic mobilities of the polypeptide chains correspond to molecular masses of 70–80 kDa. An average M_r of 75 kDa is calculated.

Table 1 shows amino acid compositions of the major *Homarus americanus* hemocyanin subunits. No considerable differences in the compositions are observed.

The N-terminal sequences of the purified subunits are compared in Table 2 together with those of other arthropodan hemocyanin subunits, published till now. Comparison of the sequences shows conserved Gln and Leu/Val/Ile residues in positions 11 and 17, respectively, in both, crustacean and cheliceratan hemocyanins. Asn 15 in all crustacean hemocyanins and Phe 18 in their cheliceratan counterparts are also conserved. Predominant Ala, Gln/His, Lys/Arg and Leu residues were observed in positions 8, 9, 10 and 18, respectively.

In the order crustacea within the infraorder palinura a high degree of N-terminal sequence identity was found for the subunit couples Pia/PjIb (65%), Pia/PjII (65%), Pia/PjIII (84%) and Pia/Pvb (63%); PjIb/PjIIb (80%), PjIb/PjIII (74%) and PjIb/Pvb (60%); PjII/PjIII (74%) and PjII/Pvb (70%) and PjIII/Pvb (74%, Table 3). This was mentioned in the paper of Neuteboom et al. [16] who reported very similar identity scores for the above discussed subunits. All these subunits are shown by Markl to belong to the α-subunit-type category according to Markl's classification [13] and the data of Neuteboom et al. [16]. The highest identity degree, 94%, is observed for the subunits Cm3/Cp1, belonging to the same infraorder and the same subunit type (α/γ), while subunits of the same type, but of different infraorders, possess lower similarity scores (CdM3′/Cm3, 54%; CdM3′/Cp1, 56%). At the same time, the identity scores between Pic (γ) and the other members within the Palinura infraorder (α; Table 3) are from 26 to 45%. Comparison between Pic and CdM1 from two different infraorders, but classified into the same subunit-type-category, γ, shows 56% identity. From Table 3 it becomes obvious that subunits, belonging to one and the same category, have relatively high identity scores ($\geq$ 50%).

Within the infraorder astacidea, the highest identity scores were observed not only between subunits from one and the same hemocyanin, like Ha1/Ha2 (63%), Ha1/Ha3 (52%), Ha1/Ha4 (63%, *H. americanus* hemocyanin) and CdM1/CdM3′ (60%, *Ch. destructor* hemocyanin), but also between polypeptide chains from different hemocyanins: *A. leptodactylus*/*H. americanus* (Alb/Ha6, 63%) and *Ch. destructor*/*H. americanus* (CdM1/Ha3, 61% and CdM3′/Ha3, 57%). On the base of the high identity between Ha1 and Cm2 (64%), between Ha2 and Cp4 (52%) and Ha4 and Cp4 (52%) on the one side, and between Ha1, Ha2 and Ha4 on the other side, these *H. americanus* subunits should be classified into one and the same subunit category, namely β. In addition, Ha2 shows a high identity degree to three palinura α-type hemocyanin subunits (PjIb, 59%; PjII, 53% and PjIII, 63%). The identity scores of the subunit couples Ha5/PjIII (58%), Ha6/Pia (52%), Ha6/Alb (63%) and Ha6/Cp1 (53%) clearly show that they belong to the α-category. Ha3 seems to belong to the α/γ-type (52% Pvb, 61% CdM1, 57% CdM3′, 54% Cm3), but it shows also similarity to Ha1 (β-type). The results are in good agreement with the data of Markl [13] who reports three immunologically different subunits in the hemocyanin of *H. americanus*.

The most primitive crustacean studied, exhibit only one subunit type and form only a hexameric structure [24]. Subunit heterogeneity and generation of 2×6 structures developed parallel to evolution—the most recent evolved crabs, the brachyurans, exhibit these features [13]. The formation of dodecamers depends on

Table 2
N-terminal amino acid sequences of arthropodan hemocyanins from three different infraorders

Phylum: Arthropoda
Order: Crustacea
Infraorders:
Palinura

	1				5				10				15				20				25				30				35			
Pia*					D	A	L	G	T	G	N	A	Q	K	**Q**	Q	D	I	**N**	H	**L**	**L**	D	K	I	**Y**	E	P	T	K	Y	P	D	-	-	**L**	K	E	I	A	E	N	F	N	P
Pib*					D	A	L	G	T	G	N	A	N	K	**Q**	Q	D	I	**N**	H	**L**	**L**	D	K	I	**Y**	E	P	T	K	Y	P	D	-	-	**L**	K	E	I	A	E	N	F	N	P
Pic*	A	D	C	Q	A	G	D	S	A	D	K	L	L	A	Q	K	**Q**	H	D	V	**N**	Y	**L**	V	Y	K	L	**Y**	G	D	I	R	D	D	H	-	-	**L**	K	E	L	G	E	T	F	N	P
PjIa*	A	D	X	Q	P	G	D	S	T	D	K	L	L	A	Q	K	**Q**	D	D	V																										
PjIb*							D	S	V	G	S	T	T	A	H	K	**Q**	Q	N	I	**N**	H	**L**	**L**	D	K																				
PjII*							D	V	V	A	S	S	T	A	H	K	**Q**	Q	D	I	**N**	H	**L**	**L**	D	K																				
PjIII*							D	A	H	G	S	G	N	A	H	K	**Q**	Q	D	I	**N**	H	**L**	**L**	D																				
Pvb*							D	V	H	S	S	D	N	A	H	K	**Q**	H	D	V	**N**	H	**L**	**L**	D	K	I	**Y**	E	P	I	K	D	E	K	-	-	**L**	H	N	T	A	H	T	F	N	-

Astacidea

Alb*							D	A	S	G	A	T	L	A	K	R	**Q**	Q	V	V	**N**	H	**L**	**L**	E	H	I	**Y**	D	H	T	H	F	T	D	-	-	**L**	K	E	I	A	E	N	F	N	P
CdM1*					D	G	S	G	G	-	S	D	A	Q	K	**Q**	H	D	V	**N**	Y	**L**	**L**	F	K	V	**Y**	E	D	V	N	D	E	N	S	P										
CdM3'*					G	V	P	G	D	V	H	D	E	Q	K	**Q**	H	D	I	**N**	F	**L**	**L**	F	K	V	**Y**	E	V	L	X	D	I	X	-	-	**L**	K	X	V	A					
Ha1								G	T	T	V	V	A	H	K	**Q**	Q	S	V	**N**	R	**L**	**L**	Y	K	V	T	S	H	I	P	D	T	F	F	S	**L**	K								
Ha2									G	A	D	V	A	H	K	**Q**	Q	S	V	**N**	H	**L**	**L**	Y	L	V	T	S	H	Y	P	S	L	D	Y	S	**L**	L								
Ha3			G	V	P	S	V	S	T	V	N	V	A	Q	K	**Q**	H	D	V	**N**	F	**L**	**L**	F	K	V	**Y**	I																		
Ha4							G	A	Y	G	Q	G	Q	N	I	G	**Q**	L	F	V	**N**	I	**L**	**I**	F	L	F	**Y**																		
Ha5							G	A	G	E	A	L	N	A	K	R	**Q**	Q	D	V	**N**	H	**L**	**L**	D	L	I	**Y**	D	H	L	H	Y													
Ha6									S	P	A	F	Q	A	Q	K	**Q**	A	K	V	**N**	D	V	**L**	D	K	L	**Y**																		

Brachyura

Cp1*							D	S	P	G	G	A	S	D	T	Q	K	**Q**	H	X	V	**N**	S	X	X	X	K	X	**Y**																		
Cp4*												A	D	L	A	H	R	**Q**	Q	S	V	**N**	R	**L**	**L**	Y	K	I	**Y**	S	P	I	S	S	A	Y	A	E	**L**	K	Q	S	T	D	N	X	D	
Cmag6*								T	A	G	G	A	F	D	A	Q	K	**Q**	H	D	V	**N**	S	A	**L**	W	K	V	**Y**	E	D	I	Q	D	P	H	L	I	Q	L	S	Q	N	F	D	P	L	S
Cm1*							X	D	P	A	S	V	S	D	A	X	K	**Q**																														
Cm2*												T	C	L	A	H	K	**Q**	Q	A	V	**N**	R	**L**	**L**	Y	R	I	**Y**	S	P	I	X	X	X	F										
Cm3*							D	S	P	G	G	A	S	D	A	Q	K	**Q**	H	D	V	**N**	S	**I**	**L**	X	K	V	**Y**	X	E	I																
Cm4									G	F	G	E	D	I	A	M	K	**Q**	H	Q	V	**N**	S	**L**	**L**	D	I	L	F	V	F																
Ms1								D	Q	P	G	D	V	K	T	H	K	**Q**	Y	D	V	**N**	Y	**L**	F	F	K	I																		
Ms2												G	Q	L	A	L	K	**Q**	Q	T	V	**N**	R	**L**	**L**	N	K	I	**Y**	S	P	I	S	D	A	Y	S	E	**L**	K	Q	L				
Ms3												G	G	P	A	G	K	**Q**	N	A	V	**N**	Q	**L**	**L**	V	L	I	**Y**	D	P	K	S	Y	K	D									
Ms4												D	G	P	A	Q	K	**Q**	N	T	V	**N**	Q	**L**	**L**	V	L	I	**Y**	L	Y	K														
Ms5							D	H	A	G	T	V	S	K	A	H	K	**Q**	H	D	V	**N**	S	V	**L**	Y	K	V	**Y**	E	D	I														
Ms6										G	G	P	S	Q	K	Q	K	**Q**	H	K	V	**N**	L	V	N	I	K	V	**Y**	I																
Ms4e												S	G	P	A	Q	K	**Q**	N	V	V	**N**	Q	**L**	**L**	V	L	I	**Y**	L	Y	K	X	D	S											

Order: Chelicerate
Infraorders:
Xiphosura

	5				10				15				20				25				30			
LiII*					T	L	H	D	K	**Q**	I	R	V	C	H	**L**	**F**	E	Q	L	S	S	A	T	V	I	G	D	G	D	K	H	K	H	S
Taa*					T	I	K	E	K	**Q**	A	S	I	L	A	**L**	**F**	E	H	L	T	S	V	P	K	-	Q	H	I	P	E	K	E	R	D		

Arachnida

An6*									T	V	A	D	K	**Q**	A	R	L	M	P	**L**	**F**	K	H	L	T	A	L	T	R	-	E	K	L	P	L	D	Q	R	D						

Araneae

Eud*													T	I	A	D	H	**Q**	A	R	I	L	P	**L**	**F**	K	K	L	T	S	L	S	P	-	D	P	L	P	E	A	E	R	D			
Eue*													P	D	K	Q	K	**Q**	L	R	V	I	S	**L**	**F**	E	H	M	T	S	I	N	T	-	-	P	L	P	R	D	Q	I	D			
Eua*												T	I	L	H	D	K	**Q**	V	Q	A	L	K	**L**	**F**	E	K	L	S	V	A	A	T	G	E	P	V	P	A	D	Q	I	D			

Pi, *Panulirus interruptus* [1,10,17]; Pj, *Panulirus japonicus* [12]; Pv, *Palinurus vulgaris* [11]; Al, *Astacus leptodactylus* [21]; Cd, *Cherax destructor* [16]; Cp, *Cancer pagurus* [16]; Cmag6, *Cancer magister* [5]; Cm, *Carcinus maenas* [16]; Li, *Limulus polyphemus* [26]; Ta, *Tachypleus tridentatus* [15]; An, *Androctonus australis* [4]; Eu, *Euripelma californicum* [19,20]; Ha, *Homarus americanus* and Ms, *Maia squinado*. Published sequences are marked by an asterisk. Completely conserved amino acid residues are printed bold.

specific subunit types involved in interactions between the two hexamers and holding them together. The presence of several subunits with very similar amino acid sequences is a general characteristic of crustacean hemocyanins and may have a role in functional flexibility and adaptation to changes in the environment. Comparison of the N-terminal sequences of 13 subunits from Brachyuran hemocyanins show high identity scores between the polypeptide chains of the *Maia squinado* hemocyanin, for the couples Ms4/Ms4e (81%), Ms4/Ms6 (70%, with the highest identities found), Ms3/Ms4e (63%), Ms3/Ms6 and Ms3/Ms4 (60%, Table 3), so these subunits must be classified into one type category. Due to the low degree of N-terminal sequence similarity of Ms1 to all other arthropodan hemocyanins listed in Table 3, no subunit category classification can be suggested. Within the Brachyura, the chain Ms2 is very similar to Cp4 (60%, *C. pagurus* hemocyanin) and Cm2 (56%, *C. maenas* hemocyanin), it is therefore reasonable to assume that these three subunits belong to the same category, the β-type. Subunit Ms5 has 61% sequence similarity to Cp1, 74% to Cm3 and 54% to Ha3 and should be classified into the α/γ-category, as well as Ms6 showing 73, 65 and 60% identity to Cp1, Cm3 and CdM1, respectively. 53% identity is found for the couple Ms4e and PjIII, reported to be immunologically related to the α-category. Based on the observed identity values between *M. squinado* subunits Ms3, Ms4 and

Table 3
N-terminal per cent identity scores between amino acid sequences of arthropod hemocyanin subunits from the two subphyla, Crustacea and Chelicerate

Pia*	Pic*	PjIb*	PjII*	PjIII*	Pvb*	Alb*	CdM1*	CdM3'*	Ha1	Ha2	Ha3	Ha4	Ha5	Ha6	Cp1*	Cp4*	Cmag6*	Cm2*	Cm3*	Cm4	Ms1	Ms2	Ms3	Ms4	Ms5	Ms6	Ms4e	LiII*	Taa*	An6*	Eud*	Eue*	Eua*	
α	γ	α	α	α	α	α	γ	α							α/γ	β		β	α/γ															
	33	**65**	**65**	**84**	**63**	**52**	41	37	30	38	44	30	33	**52**	41	33	48	50	33	26	43	37	37	33	33	33	42	15	15	15	15	11	15	Pia*
		35	40	37	45	26	**56**	37	33	27	48	22	22	26	47	33	44	33	41	26	43	37	22	26	41	33	38	22	15	22	19	19	22	Pic*
			80	**74**	**60**	50	45	35	50	**59**	35	20	40	39	31	50	35	50	35	42	40	50	38	38	35	28	38	33	40	33	40	20	25	PjIb*
				74	**70**	40	45	40	50	**53**	45	20	45	39	31	50	35	50	40	37	40	50	38	38	40	28	44	27	27	27	33	20	25	PjII*
					74	**53**	42	37	47	**63**	38	32	38	35	27	47	42	**53**	42	44	42	47	47	47	37	22	**53**	29	29	29	29	21	20	PjIII*
						37	48	41	41	43	**52**	37	44	44	37	**52**	48	41	41	37	43	44	33	33	48	41	42	19	15	15	15	15	15	Pvb*
							38	22	30	46	35	41	30	**63**	35	48	36	37	30	26	33	37	33	30	26	33	42	30	19	22	15	19	15	Alb*
								60	41	32	**61**	33	40	37	**76**	30	**72**	30	**71**	33	48	37	30	33	**64**	**60**	42	15	11	15	15	22	15	CdM1*
									33	27	**57**	22	32	30	**56**	22	**52**	22	**54**	22	37	30	22	26	48	43	35	11	19	11	15	19	15	CdM3'*
										63	**52**	**63**	41	33	33	48	44	**64**	37	26	42	**48**	26	26	48	30	33	19	22	22	26	22	19	Ha1
											40	32	46	37	40	**52**	36	50	41	43	50	34	36	38	41	35	33	25	21	29	21	25	14	Ha2
												27	35	50	53	42	**52**	42	**54**	36	38	47	42	47	**54**	48	47	28	17	28	22	22	21	Ha3
													33	41	29	**52**	22	41	33	33	30	33	33	30	33	30	39	22	22	22	22	22	11	Ha4
														30	35	30	40	30	48	30	33	33	30	33	48	33	35	15	11	19	15	19	15	Ha5
															53	41	**55**	33	33	26	26	22	41	33	33	33	44	11	8	11	11	11	8	Ha6
																46	**76**	38	**94**	44	44	46	38	46	**61**	**73**	46	25	17	17	17	50	23	Cp1*
																	36	**73**	33	26	41	**60**	37	30	37	37	33	19	15	11	19	15	15	Cp4*
																		45	**75**	39	33	40	28	38	**68**	**52**	38	12	12	16	16	28	16	Cmag6*
																			30	26	41	**56**	41	33	37	33	38	22	15	15	15	19	19	Cm2*
																				37	29	33	26	30	**74**	**65**	38	11	7	11	11	22	11	Cm3*
																					30	26	26	26	30	26	35	19	19	19	19	19	22	Cm4
																						41	35	41	38	32	35	25	25	25	25	31	24	Ms1
																							44	37	33	37	42	22	15	15	19	19	19	Ms2
																								60	26	**60**	**63**	15	11	15	11	15	11	Ms3
																									26	**70**	**81**	15	11	15	11	19	11	Ms4
																										26	38	11	7	11	11	15	11	Ms5
																											32	15	11	15	11	19	11	Ms6
																												20	15	15	10	20	15	Ms4e
																													30	33	30	30	41	LiII*
																														33	**48**	48	30	Taa*
																															56	33	30	An6*
																																33	33	Eud*
																																	30	Eue*
																																		Eua*

Pi, *Panulirus interruptus* [1,10,17]; Pj, *Panulirus japonicus* [12]; Pv, *Palinurus vulgaris* [11]; Al, *Astacus leptodactylus* [21]; Cd, *Cherax destructor* [16]; Cp, *Cancer pagurus* [16]; Cmag6, *Cancer magister* [5]; Cm, *Carcinus maenas* [16]; Li, *Limulus polyphemus* [26]; Ta, *Tachypleus tridentatus* [15]; An, *Androctonus australis* [4]; Eu, *Euripelma californicum* [19,20]; Ha, *Homarus americanus* and Ms, *Maia squinado*. Published sequences are marked by an asterisk. α-, β- and γ-subunit type classification is given in Neuteboom et al. [16]. Identity scores higher than 50% are printed bold. *Panulirus interruptus* b (Pib) is not included since it is identical to *Panulirus interruptus* a (Pia); *Panulirus japonicus* 1a (PjIa) and *Carcinus maenas* 1 (Cm1) are not included since their sequences are too short. Residues 1–25, if shorter, as far as determined, are compared. The subunits are designated by a, b, c, d, e, by 1, 2, 3, 4, 5, 6, or II, indicating the peak labeling in the isolation profile. No insertions or deletions are considered.

Ms4e, Ms3 and Ms4 should be put into the same category. Within the brachyura, Cmag6 shows a high similarity value when compared with Cp1 (76%), Cm3 (75%), Ms5 (68%), Ms6 (52%) and should be considered as α/γ-type subunit. This is supported by a comparison of Cmag6 with hemocyanin subunits from astacidea, CdM1 (72%), CdM3′ (52%) and Ha3 (52%), belonging also to the α/γ-category.

With N-terminal sequences of chains from different crustacean infraorders high similarity scores are found for the comparison of *Ch. destructor* M1 (CdM1) with *C. pagurus* Cp1 (76%), *C. magister* Cmag6 (72%) and *C. maenas* Cm3 (71%), as well as with *Maia squinado* Ms5 (56%), Ms6 (60%) and *P. interruptus* Pic (56%). These seven subunits were considered to be closely related and grouped in the α/γ-category. 52% and 53% identity is observed between the N-terminal sequences of the couples Pia/Alb and PjIII/Alb, all of them classified as α-type subunits. The high similarity scores found within subunits of the three crustacean infraorders lead to the conclusion, that the hemocyanins of the studied crustacean infraorders must have resulted from a gene duplication of a common ancestral arthropod binuclear copper protein, which occurred before the divergence between brachyura, astacidea and palinura.

In the order chelicerate no identity scores higher than 50% were calculated for the subunits of xiphosuran hemocyanins. Within the infraorder arachnida, 56% sequence similarity was found for the subunits Eud (*E. californicum* hemocyanin) and An6 (*A. australis* hemocyanin). It should be mentioned that the degree of identity, calculated from the N-terminal sequences, is usually lower than that determined from the complete amino acid sequences. Thus, for the subunits An6/Eue and An6/Taa, the N-terminal sequence analysis gives a value of 33% identity while comparison of the complete sequences [2,16] of the same subunits shows 64–65% sequence similarity.

The crustaceans and chelicerates diverged at least 600 million years ago from a common ancestral copper-binding protein by sequence duplication [13,24]. Both phyla exhibit a common hexameric hemocyanin structure, an evidence, that it must have been established before this division. Further evidence comes from immunological cross-reactivity of chelicerate and crustacean hemocyanins [13]. However, the N-terminal primary structures of crustacean and cheliceratan hemocyanins are not related to each other and show only a few identities. Complete amino acid sequences of hemocyanins from the two subphyla demonstrate identity around 33% [8]. This is confirmed when the N-terminal parts of the structural subunits are compared: an identity between 7 and 40% is obtained only (Table 3). In some cases, the sequence similarity between the N-terminal sequences of subunits from one and the same hemocyanin is also very low, supporting the conclusion that the N-terminal sequences of arthropod hemocyanins are the most variable part of their polypeptide chains and not suited for phylogenetic studies [3].

Our results demonstrate that independent evolution is possible not only for members of different subphyla but also for those from one infraorder or of the same hemocyanin.

In conclusion, the results demonstrate, that N-terminal sequence analysis can be used to discriminate between different subunit types not only within infraorders, but also between subunits from different infraorders. However, in a few cases N-terminal sequence comparison offers no reliable and clear classification into a distinct category. N-terminal sequence analysis in combination with sensitive specific immunoblotting, applied systematically to appropriate selected hemocyanins, can provide more reliable information for a subunit type classification.

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References

[1] Bak HJ, Beintema JJ. *Panulirus interruptus* hemocyanin: The elucidation of the complete amino acid sequence of subunit a. Eur J Biochem 1987;169:333–48.
[2] Beintema JJ, Stam WT, Hazes B, Smidt MP. Evolution of arthropod hemocyanins and insect storage proteins (hexamerins). Mol Biol Evol 1994;11:493–503.
[3] Burmester T, Scheller K. Common origin of arthropod tyrosinase, arthropod hemocyanin, insect hexamerin and dipteran arylphorin receptor. J Mol Evol 1996;42:713–28.
[4] Buzy A, Gagnon J, Lamy J, Thibault P, Forest E, Hudry-Clergeon G. Complete amino acid sequence of the Aa6 subunit of the scorpion *Androctonus australis* hemocyanin determined by Edman degradation and mass spectrometry. Eur J Biochem 1995;233:93–101.
[5] Durstewitz G, Terwilliger NB. cDNA cloning of a developmentally regulated hemocyanin subunit in the crustacean Cancer magister and phylogenetic analysis of the hemocyanin gene family. Mol Biol Evol 1997;14:266–76.
[6] Ellmann GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82:70–7.
[7] Ghiretti-Magaldi A, Ghiretti F. The pre-history of hemocyanin. The discovery of copper in the blood of molluscs. Experientia 1992;48:971–2.
[8] Hazes B, Magnus KA, Bonaventura C, Bonaventura J, Dauter Z, Kalk KH, Hol WGJ. Crystal structure of deoxygenated *Limulus polyphemus* subunit II hemocyanin at 2.18 Å resolution: clues for a mechanism for allosteric regulation. Protein Sci 1993;2:597–619.
[9] Herskovits TT. Recent aspects of the subunit organization and dissociation of hemocyanins. Comp Biochem Physiol 1988;91B:597–611.

[10] Jekel PA, Bak HJ, Soeter NM, Vereijken JM, Beintema JJ. *Panulirus interruptus* hemocyanin: The amino acid sequence of subunit b and anomalous behaviour of subunits a and b on polyacrylamide gel electrophoresis in the presence of SDS. Eur J Biochem 1988;178:403–12.

[11] Jekel PA, Neuteboom B, Beintema JJ. Primary structure of hemocyanin from *Palinurus vulgaris*. Comp Biochem Physiol 1996;115B:243–6.

[12] Makino N, Kimura S. Subunits of *Panulirus Japonicus* hemocyanin. 1. Isolation and properties. Eur J Biochem 1988;173:423–30.

[13] Markl J. Evolution and function of structurally diverse subunits in the respiratory protein hemocyanin from arthropods. Biol Bull 1986;171:90–115.

[14] Markl J, Decker H. Molecular structure of the arthropod hemocyanins. In: Mangum CP, editor. Adv Comp Environ Physiol, vol. 13. Heidelberg: Springer, 1992:325–76.

[15] Nemoto T, Takagi T. Primary structure of *Tachypleus tridentatus* hemocyanin α chain. In: Wood JE, editor. Structure and Function of Invertebrate Respiratory Proteins. Life Chem Rep Suppl 1. Harwood, London, 1983:89–92.

[16] Neuteboom B, Sierdsema SJ, Beintema JJ. The relationship between N-terminal sequences and immunological characterization of Crustacean hemocyanins. Comp Biochem Physiol 1989;94B:587–92.

[17] Neuteboom B, Jekel PA, Beintema JJ. Primary structure of hemocyanin subunit c from Panulirus interruptus. Eur J Biochem 1992;206:243–9.

[18] Ricchelli F, Jori G, Tallandini L, Zatta P, Beltramini M, Salvato B. The role of copper and quaternary structure on the conformational properties of *Octopus vulgaris* hemocyanin. Arch Biochem Biophys 1984;235:461–9.

[19] Schartau W, Eyerle F, Reisinger P, Geisert H, Storz H, Linzen B. Hemocyanins in spiders. XIX. Complete amino acid sequence of subunit d from Eurypelma californicum hemocyanin and comparison to chain e. Hoppe-Seyler's Z Physiol Chem 1983;364:1383–409.

[20] Schneider HJ, Drexel R, Feldmaier G, Linzen B, Lottspeich F, Henschen A. Hemocyanins in spiders. XVIII. Complete amino acid sequence of subunit e from *Euripelma californicum* hemocyanin. Hoppe-Seyler's Z Physiol Chem 1983;364:1357–81.

[21] Schneider HJ, Voll W, Lehmann G, Grisshammer R, Goettgens A, Linzen B. Partial amino acid sequence of crayfish (*Astacus leptodactylus*) hemocyanin. In: Linzen B, editor. Invertebrate Oxygen Carriers. Berlin: Springer, 1986:173–6.

[22] Stöcker W, Räder U, Bijlholt MMC, Wichertjes T, van Bruggen EFJ, Markl J. The quaternary structure of four Crustacean two-hexameric hemocyanins: immunocorrelation, stoichiometry, reassembly and topology of individual subunits. J Comp Physiol 1988;158B:271–89.

[23] van Holde KE, Miller KI, Lang WH. Molluscan hemocyanins: structure and function. Adv Comp Envir Physiol 1992;13:258–300.

[24] van Holde KE, Miller KI. Hemocyanins. Adv Prot Chem 1995;47:1–81.

[25] Volbeda A, Hol WGJ. Crystal structure of hexameric haemocyanin from *Panulirus interruptus* refined at 3.2 Å resolution. J Mol Biol 1989;209:249–79.

[26] Yokota E, Riggs AF. The structure of the hemocyanin from the Horseshoe crab, *Limulus polyphemus*. J Biol Chem 1984;259:4739–4749; Jekel PA, Bak HJ, Soeter NM, Vereijken JM, Beintema JJ. *Panulirus interruptus* hemocyanin: The amino acid sequence of subunit b and anomalous behaviour of subunits a and b on polyacrylamide gel electrophoresis in the presence of SDS. Eur J Biochem 1988;178:403–412.