

Molecular cloning of hemocyanin cDNA from *Penaeus vannamei* (Crustacea, Decapoda): structure, evolution and physiological aspects

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Abstract Hemocyanin is present as 2 subunits in the hemolymph of *Penaeus vannamei*. Isolated from a hepatopancreas cDNA library of this penaeid shrimp, the cDNA chain (2095 bp) corresponds to a full length hemocyanin messenger as determined by Northern hybridization, with a short 5' untranslated region (17 bp), an open reading frame (1989 bp counting initiation and termination codons) coding for a signal peptide (13 residues) and a mature hemocyanin (648 amino acids), and a 3' untranslated region (89 bp) followed by the polyadenylated track. It is the first time that the existence of a hydrophobic signal peptide is shown in arthropod hemocyanin. Two primary N-terminal sequences are determined and a 3-fold increase of mRNA content, measured in the hepatopancreas during the premoult stages, is reported. The low level of polymorphism shown by *P. vannamei* hemocyanin, along with its weak percentage identity with counterparts and its similarity with hemocyanin from *Panulirus interruptus*, suggests that this arthropod hemocyanin may be a primitive subunit that has evolved independently, following gene duplication.

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Key words: Hemocyanin; cDNA nucleotide sequence; Invertebrate; Crustacea

1. Introduction

Hemocyanin is a copper-containing respiratory pigment assuming O₂ transport, colorless when de-oxygenated, but appearing light blue when oxygenated. Hemocyanins occur in two phyla of invertebrates: the arthropoda [1–6] and the mollusca [7], occurring in several different classes: Crustacea, Myriapoda (Chilopoda), Merostomata (horseshoe crab) and Arachnida. As the main protein component of hemolymph, hemocyanin typically represents up to 95% of the total amount of protein. In crustacean hemolymph, several components differing in size, as estimated by their sedimentation coefficients ranging from 39 S to 16 S (as it is in *Palimurus* or *Penaeus*) were determined [8,9]. The hexamer, with a molecular mass of 4.5×10^5 Da, is the predominant form in the most primitive crustacean Decapoda such as *Penaeus setiferus* [9] or *P. monodon* [10]. For the mean value of the smallest functional component, the polypeptide monomers contain 600–660 amino acid residues depending on species (625 for chelicerate hemocyanins and 660 for crustacean protein). Between three and eight different subunits, classified on immunological data, have been distinguished in crustacean hemocyanin [11]. Alpha type is present in all of them, but beta and

gamma types may be absent [12]. The hepatopancreas has been shown to be the site of hemocyanin synthesis [13]. Until now, only several partial N-terminal and some complete amino acid sequences of the different subunits from *Panulirus interruptus* were determined in crustacea [14–16], and no cDNA sequence was further characterized. Hemocyanin cDNAs were isolated from the tarantula *Eurypelma californicum* [17] and from the mollusk *Octopus dofleini* [7]. Surprisingly, no leader sequence coding for a signal peptide was detected in the hemocyanin gene in tarantula [18]. The authors argue that the secretion of this extracellular pigment is achieved by the accumulation of this protein in the cytoplasm released by holocrine secretion.

In this paper, we report on the purification and electrophoretic characterization of *Penaeus vannamei* hemocyanin and on the molecular cloning and complete sequencing of one hemocyanin cDNA from the hepatopancreas. The presence in the deduced protein sequence of an hydrophobic signal peptide, absent in the other invertebrate hemocyanins, is discussed in relation to differences in the mode of secretion of this protein in relation to the penaeid moulting cycle.

2. Materials and methods

Shrimps (*Penaeus vannamei*) were obtained from Ifremer (Tahiti). The hepatopancreas was removed by dissection, immediately frozen in liquid nitrogen and stored at -80°C . Determination of the moulting stages was made according to the method of Drach and Tchernigotzeff [19].

2.1. Hemocyanin and RNA extractions and purifications

Hemocyanin was taken in a 1 ml syringe by puncturing the heart. After coagulation, the clot was disrupted by passage through the needle and the sample was centrifuged 5 min at $14000 \times g$. Hemocyanin complex was obtained from the serum of one animal by chromatography on a Sephadex G100 column (15×300 mm). Proteins from the main peak eluted in the exclusion volume of the column were characterized by electrophoresis on a 3% stacking gel and 7% SDS-PAGE acrylamide gel [20]. An aliquot of the G100 purified hemocyanin was used for the determination of the N-terminal amino acid sequence [21].

Total RNA extractions were made following the guanidine thiocyanate method [22] after disruption of the digestive glands in liquid nitrogen. Poly-A⁺ RNAs were purified by affinity chromatography on oligo dT-cellulose column. Total RNA was isolated from 50 mg fresh weight individual hepatopancreas from 2–6 shrimps for each of the different moulting stages using the rapid RNA extraction kit from Stratagene. A 256 bp *EcoRI* coding fragment of the shrimp hemocyanin cloned cDNA was used to probe RNA, which was fractionated by denaturing electrophoresis on 1.5% agarose gels in phosphate buffer. Transfer to nylon membrane (Hybond N⁺, Amersham) was performed as described by the manufacturer. Dots of known RNA amounts of each of the moulting stages, quantified by spectrophotometry, were directly fixed on membranes to estimate variation of the hemocyanin mRNA during the moulting cycle. The membranes were treated as for the screening procedure.

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EBI sequence accession number: X82502.

2.2. cDNA library screening

A lambda ZAP cDNA library for shrimp hepatopancreas was established following the manufacturer's protocol (Stratagene, La Jolla, CA, USA). The amplified cDNA library, containing 8×10^9 phages per ml, was first used to select randomly 15 isolated clones among 500 phage plaques. In a second step, plaques were transferred to Hybond N⁺ membranes (Amersham) and screened using a 5' end 498 bp *EcoRI* fragment of the cloned cDNA we obtained for the first selection as a probe covering nucleotides 1262–1759. Prehybridization of the duplicate membranes was achieved in a 50% formamide solution containing 1% sodium dodecyl sulfate (SDS), 1 M NaCl and 100 µg yeast RNA per ml for 4 h at 42°C. For hybridization, the denatured probe was added (10^6 cpm/ml and 8×10^8 cpm/µg) for 16 h at the same temperature. The filters were washed twice in $2 \times$ sodium saline citrate (SSC) for 5 min at room temperature, then twice in $2 \times$ SSC containing 1% SDS at 65°C for 30 min, and finally twice in $0.1 \times$ SSC for 30 min at room temperature.

In order to have complete cDNAs, a third screening was performed using a synthetic oligonucleotide (CATGAGTGCCTCATTAC-GATGCC) based on the 5' end of the longest previously isolated cDNA hybridizing to nucleotides 275–297, end-labelled with [γ -³²P]ATP [23].

2.3. Plasmid subcloning and sequencing

The existence of five restriction sites for *EcoRI* and two for *Bam*HI in the complete cDNA (clone Hemo20) allow us to obtain six subclones in pBluescript SK and KS minus (Stratagene) covering the whole insert. The two *Bam*HI restriction sites in position 762 and 1107 were used to generate a subclone encompassing the portion containing the two close by *EcoRI* sites. All these subclones were sequenced in the both orientations. In the case of the large *EcoRI* fragment (657 bp), two internal primers were used (the one used for the screening on the complementary strand and another one with sequence CCAATTCCTTTGATCCCGTT (hybridizing to nucleotide 143–162, on the coding strand). DNA was sequenced following the dideoxynucleotide method with modification for extended DNA sequencing. Electrophoresis of the extended products was performed with two or three successive loadings on a 5% acrylamide/bisacrylamide (30/0.8) gel.

2.4. Extension of 5' mRNA end

Specific partial hemocyanin cDNAs were synthesized using a RACE 5'/3' kit (Boehringer Mannheim) from total RNA with a specific anti-sense oligonucleotide: CATGAGTGCCTCATTAC-GATGCC hybridizing to nucleotides 275–297. The 5' end of the hemocyanin cDNA was amplified using an oligo-dT anchor primer hybridizing to the appended poly-A tail and a second specific anti-sense oligonucleotide (HEMO2R): CCATCACTGTAGGTGCC-TAAGTTTC, hybridizing to nucleotides 164–188.

3. Results and discussion

3.1. Hemocyanin characterization

Hemocyanin from *Penaeus vannamei* was observed as two close prominent bands of protein with molecular weights around 73 and 75 kDa as determined by SDS-denaturing gel electrophoresis (Fig. 1). These results confirm the work of Chan et al. [24] and similar results have been recorded for *Penaeus setiferus* [8] and *Penaeus semisculatus* [25]. The bands represent the monomeric polypeptides of hemocyanin and suggest subunit heterogeneity.

3.2. Library screening and hemocyanin cDNA characterization

By screening the cDNA library, approximately 8% of the phage plaques were found to be positive. Further screening with the oligonucleotide probe allowed us to isolate incomplete cDNAs. Using the 5' RACE method with Pfu DNA polymerase (Stratagene) and the reverse oligonucleotide HEMO2R, a 250 bp fragment was obtained and cloned. The analysis of these cloned inserts confirmed the full length sequence of the cDNA.

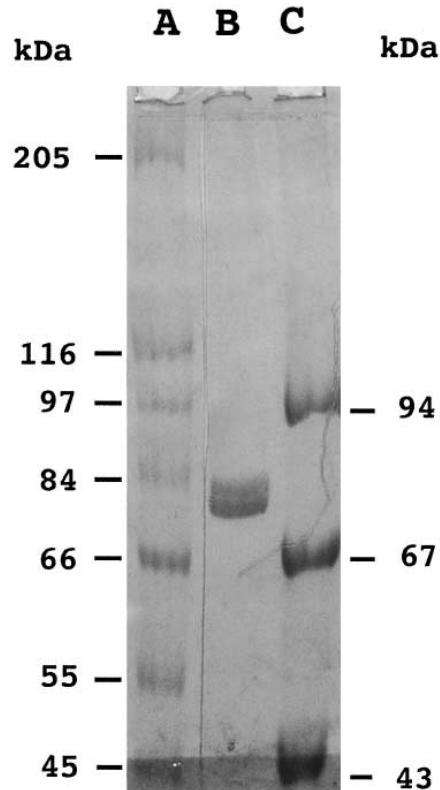


Fig. 1. SDS-denaturing electrophoresis of *Penaeus vannamei* hemocyanin on a 7% acrylamide/bisacrylamide gel. 4 µg of protein was denatured and loaded on the gel. After electrophoresis, proteins were stained with Coomassie blue. A: High range markers (Sigma); myosin (205 000), β -galactosidase (116 000), phosphorylase b (97 000), fructose-6-phosphate kinase (84 000), bovine serum albumin (66 000), glutamic dehydrogenase (55 000) and ovalbumin (43 000). B: Crustacean hemocyanin sample. C: Low molecular weight markers (Pharmacia); phosphorylase b (94 000), albumin (67 000) and ovalbumin (43 000).

The longest, complete cDNA sequence recorded is 2095 bp long (without the poly-A tail), including a 17 bp 5' untranslated region, a 1989 bp coding sequence (counting initiation and termination codons) and an 89 bp untranslated 3' end with the polyadenylated track (Fig. 2). The translation initiation site bears no resemblance to a Kozak motif [26] and does not contain an in-frame stop sequence. Moreover the presence of the very short 5' untranslated extension has been observed for major digestive enzymes such as trypsin [27], cathepsin [28] and amylase [29], synthesized and secreted in the penaeid digestive gland. Such an extension is also found in prophenoloxidase (a member of the hemocyanin family) in *Pacifastacus leniusculus* [30]. The open reading frame encodes 661 amino acids forming a preprotein composed of a hydrophobic signal peptide (13 residues; not observed in tarantula [18]) and a mature protein (648 amino acids). This hydrophobic signal sequence ends with a stretch of 4 alanines corresponding to an Ala-X-Ala motif, a frequent consensus preceding the cleavage site of signal peptides [31]. Given that hemocyanin should be stored in secretory vesicles as has been observed for digestive enzymes in the F cells of the crustacean hepatopancreas [27–29], the presence of this sequence, absent in the other known invertebrate hemocyanins, implies that the crustacean hemocyanin passes through the endoplasmic reticulum membrane.

ATG	AGG	GTC	TTA	GTG	GTT	CTT	GGG	CTT	GTC	GCT	GCT	GCC	GCC	TTT	CAG	GTG	GCC	AGT	GCA	GAT	GTT	CAG	CAG	CAG	AAA	78	
Met	R	V	L	V	V	L	G	L	V	A	A	A	A	F	Q	V	A	S	A	D	V	Q	Q	Q	K	12	
GAC	GTC	CTT	TAT	CTC	CTG	AAC	AAA	ATC	TAT	GGA	GAC	ATT	CAG	GAC	GGA	GAC	CTG	CTG	GCT	ACT	<u>GCC</u>	<u>AAT</u>	<u>TCC</u>	<u>TTT</u>	<u>GAT</u>	156	
D	V	L	Y	L	L	N	K	I	Y	G	D	I	Q	D	G	D	L	L	A	T	A	N	S	F	D	38	
<u>CCC</u>	<u>GTT</u>	<u>GGA</u>	<u>AAC</u>	<u>TTA</u>	<u>GGC</u>	<u>AGC</u>	<u>TAC</u>	<u>AGT</u>	<u>GAT</u>	<u>GGT</u>	GCA	GCC	GTG	CAA	AAA	CTG	GTG	CAG	GAC	CTT	AAT	GAC	GGC	AAA	234		
P	V	G	N	L	G	S	Y	S	D	G	G	A	A	V	Q	K	L	V	Q	D	L	N	D	G	K	64	
CTC	TTG	GAG	CAG	AAA	CAC	TGG	TTC	TCC	CTT	TTC	AAT	ACA	<u>AGG</u>	<u>CAT</u>	<u>CGT</u>	<u>AAT</u>	<u>GAG</u>	<u>GCA</u>	<u>CTC</u>	<u>ATG</u>	CTT	TTC	GAC	GTC	CTC	312	
L	L	E	Q	K	H	W	F	S	L	F	N	T	R	H	R	N	E	A	L	M	L	F	D	V	L	90	
ATC	CAC	TGC	AAA	GAC	TGG	GCA	TCC	TTT	GTC	GGC	AAT	GCA	GCC	TAC	TTC	CGT	CAG	AAA	ATG	AAC	GAA	GGA	GAG	TTT	GTT	390	
I	H	C	K	D	W	A	S	F	V	G	N	A	A	Y	F	R	Q	K	M	N	E	G	E	F	V	116	
TAT	GCC	TTG	TAT	GTT	GCC	GTG	ATC	CAC	TCG	TCT	TTG	GCT	GAA	CAG	GTG	GTG	CTC	CCT	CCC	CTC	TAT	GAG	GTC	ACA	CCG	468	
Y	A	L	Y	V	A	V	I	H	S	S	L	A	E	Q	V	V	L	P	P	L	Y	E	V	T	P	142	
CAC	CTC	TTC	ACC	AAC	AGT	GAA	GTT	ATC	GAA	GAA	GCT	TAT	CGT	GCC	AAA	CAG	AAG	CAG	ACG	CCT	GGC	AAA	TTC	AAG	TCC	546	
H	L	F	T	N	S	E	V	I	E	E	A	Y	R	A	K	Q	K	Q	T	P	G	K	F	K	S	168	
TCC	TTT	ACG	GGA	ACC	AAG	AAA	AAC	CCT	GAA	CAG	AGA	GTG	GCA	TAT	TTC	GGT	GAA	GAT	ATC	GGC	TTG	AAT	ACC	CAT	CAC	624	
S	F	T	G	T	K	K	N	P	E	Q	R	V	A	Y	F	G	E	D	I	G	L	N	T	H	H	194	
GTT	ACC	TGG	CAT	ATG	<u>GAA</u>	<u>TTC</u>	CCC	TTC	TGG	TGG	AAT	GAT	GCT	TAC	GGC	CAT	CAT	CTG	GAT	CGC	AAA	GGA	GAA	AAC	TTC	702	
V	T	W	H	M	E	F	P	F	W	W	N	D	A	Y	G	H	H	L	D	R	K	G	E	N	F	220	
TTC	TGG	ATT	CAT	CAC	CAA	CTT	ACC	GTC	CGA	TTT	GAT	GCT	GAA	CGT	CTG	TCC	AAT	TAT	CTG	<u>GAT</u>	<u>CCA</u>	GTA	GGT	GAA	CTC	780	
F	W	I	H	H	Q	L	T	V	R	F	D	A	E	R	L	S	N	Y	L	D	P	V	G	E	L	246	
CAG	TGG	AAC	AAG	CCC	ATT	GTA	GAT	GGC	TTT	GCT	CCC	CAC	ACC	ACT	TAC	AAG	TAT	GGA	GGT	CAG	TTC	CCT	GCT	CGT	CCT	858	
Q	W	N	K	P	I	V	D	G	F	A	P	H	T	T	Y	K	Y	G	G	Q	F	P	A	R	P	272	
GAC	AAT	GTT	AAA	TTC	GAA	GAT	GTG	GAC	GAT	GTT	GCT	<u>CGA</u>	<u>ATT</u>	<u>CGA</u>	GAT	ATG	GTC	ATC	GTG	GAG	AGT	<u>CGA</u>	<u>ATT</u>	CGT	GAT	936	
D	N	V	K	F	E	D	V	D	G	D	V	A	R	I	R	D	M	V	I	V	E	S	R	I	R	D	298
GCC	ATT	GCC	CAT	GGC	TAT	ATA	GTT	GAC	AGT	GAG	GGC	AAA	CAC	ATT	GAC	ATC	AGT	AAT	GAG	AAA	GGT	ATT	GAC	ATT	CTT	1014	
A	I	A	H	G	Y	I	V	D	S	E	G	K	H	I	D	I	S	N	E	K	G	I	D	I	L	324	
GGT	GAT	ATC	ATC	GAA	TCC	TCA	CTA	TAC	AGT	CCC	AAC	GTG	CAG	TAC	TAT	GGA	GCT	TTA	CAT	AAC	ACT	GCC	CAT	ATT	GTA	1092	
G	D	I	I	E	S	S	L	Y	S	P	N	V	Q	Y	Y	G	A	L	H	N	T	A	H	I	V	350	
CTA	GGC	CGT	CAA	GGG	<u>GAT</u>	<u>CCT</u>	CAT	GGA	AAG	TTT	GAT	TTA	CCA	CCT	GGT	GTG	CTG	GAA	CAC	TTC	GAA	ACT	GCC	ACC	CGT	1170	
L	G	R	Q	G	D	P	H	G	K	F	D	L	P	P	G	V	L	E	H	F	E	T	A	T	R	376	
GAT	CCC	AGC	TTC	TTT	CGG	CTT	CAC	AAG	TAT	ATG	GAT	AAC	ATT	TTC	AAA	GAA	CAC	AAG	GAC	AAC	CTA	CCC	CCA	TAC	ACC	1248	
D	P	S	F	F	R	L	H	K	Y	M	D	N	I	F	K	E	H	K	D	N	L	P	P	Y	T	402	
AAA	GCC	GAT	TTG	<u>GAA</u>	<u>TTC</u>	TCT	GGC	GTG	TCT	GTC	ACA	GAG	CTA	GCC	GTT	GTA	GGT	GAA	CTG	GAG	ACC	TAC	TTT	GAA	GAT	1326	
K	A	D	L	E	F	S	G	V	S	V	T	E	L	A	V	V	G	E	L	E	T	Y	F	E	D	428	
TTT	GAA	TAC	AGT	CTT	ATC	AAC	GCA	GTT	GAT	GAT	GCT	GAA	GGA	ATC	CCA	GAT	GTG	GAA	ATC	AGC	ACA	TAT	GTG	CCT	CGT	1404	
F	E	Y	S	L	I	N	A	V	D	D	A	E	G	I	P	D	V	E	I	S	T	Y	V	P	R	454	
CTT	AAC	CAC	AAA	GAG	TTC	ACT	TTT	AGG	ATT	GAT	GTA	GAG	AAT	GGA	GGT	GCT	GAG	AGA	TTG	GCT	ACA	GTT	CGT	ATC	TTT	1482	
L	N	H	K	E	F	T	F	R	I	D	V	E	N	G	G	A	E	R	L	A	T	V	R	I	F	486	
GCC	TGG	CCT	CAT	AAA	GAC	AAC	AAC	GGA	ATC	GAG	TAT	ACA	TTT	GAC	GAA	GGT	CGC	TGG	AAT	GCC	ATC	GAG	TTG	GAT	AAG	1560	
A	W	P	H	K	D	N	N	G	I	E	Y	T	F	D	E	G	R	W	N	A	I	E	L	D	K	506	
TTC	TGG	GTA	TCT	TTG	AAG	GGT	GGA	AAA	ACT	TCA	ATT	GAA	CGC	AAG	TCC	ACG	GAA	TCT	TCA	GTA	ACT	GTA	CCG	GAC	GTG	1638	
F	V	S	L	K	G	G	K	T	S	I	E	R	K	S	T	E	S	V	T	V	T	P	D	V	532		
CCA	AGC	ATA	CAT	GAC	CTG	TTT	GCA	GAA	GCC	GAG	GCA	GGC	GGC	GCT	GGC	CTT	GCC	AAA	TTC	GAG	AGT	GCA	ACA	GGC	CTA	1716	
P	S	I	H	D	L	F	A	E	A	E	A	G	G	A	G	L	A	K	F	E	S	A	T	G	L	558	
CCA	AAC	AGG	TTC	CTT	CTC	CCC	AAG	GGC	AAC	GAT	AGA	GGC	CTG	<u>GAA</u>	<u>TTC</u>	GAC	CTT	GTG	GTG	GCG	GTG	ACT	GAT	GGT	GAT	1794	
P	N	R	F	L	L	P	K	G	N	D	R	G	L	E	F	D	L	V	V	A	V	T	D	G	D	584	
GCC	GAC	TCA	GCA	GTG	CCG	AAC	CTT	CAT	GAG	AAT	ACC	GAG	TAC	AAT	CAC	TAC	GGT	TCC	CAT	GGC	GTG	TAC	CCC	GAT	AAG	1872	
A	D	S	A	V	P	N	L	H	E	N	T	E	Y	N	H	Y	G	S	H	G	V	Y	P	D	K	610	
CGT	CCT	CAT	GGT	TAT	CCT	CTG	GAC	CGC	AAA	GTT	CCA	GAT	GAG	CGC	GTG	TTT	GAA	GAT	CTT	CCT	AAC	TTT	AAG	CAC	ATC	1950	
R	P	H	G	Y	P	L	D	R	K	V	P	D	E	R	V	F	E	D	L	P	N	F	K	H	I	636	
CAA	GTT	AAG	GTC	TTC	AAT	CAT	GGT	GAA	CAC	ATT	CAT	TAG	TGATTCAAGCTTACAACCTCTTCATATGAATATGTCGATGAATGAATTGTTT	2040													
Q	V	K	V	F	N	H	G	E	H	I	H	Ter	648														
CTTTGAATTATTTCCCAATAAAGAACATATGCATAC(A)n													2078														

Fig. 2. Nucleotide sequence of shrimp hepatopancreas hemocyanin cDNA and deduced amino acid sequence. The amino acid sequence is numbered sequentially from the first amino acid of the mature protein. The proposed signal peptide is represented by amino acids –13 to –1 (bold letters). The hybridization sites of the three oligonucleotide probes used for the screening, the sequencing and the 5' extension are underlined. The proposed polyadenylation site is also underlined. Restriction sites for *EcoRI* and *BamHI* and initiation and termination codons are shown in bold letters. Histidines involved in the two copper binding sites are marked with asterisks.

Hemocyanin typically shows a high degree of polymorphism, based primarily on electrophoretic migration or immunoreactivity (see Markl and Decker [32] or Van Holde and Miller [33] for a review). In the tarantula and in *Panulirus interruptus* several hemocyanin sequences can be established

or deduced from cDNAs. However, using our *Penaeus vannamei* cDNA library, during the first random selection and the two further screenings, around 50 clones were isolated and the inserts were characterized and partially sequenced. All these cDNAs showed the same nucleotide alignment with the ex-

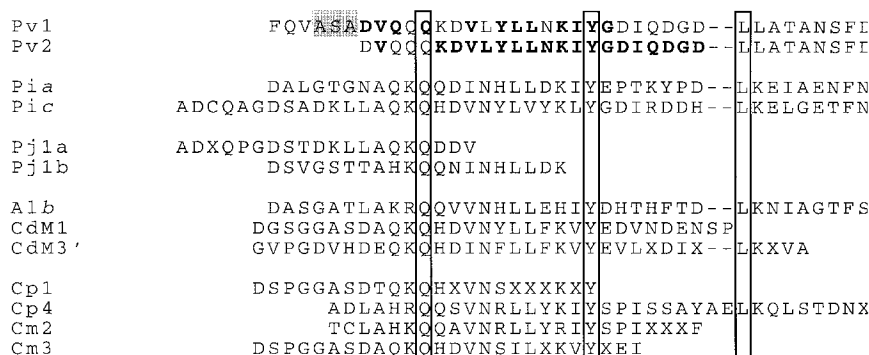


Fig. 3. Alignment of N-terminal partial amino acid sequences of crustacean hemocyanins. The proposed deletions in *P. vannamei* (Pv) compared to the other sequences are indicated by deletion symbols (—). Undetermined residues are indicated by X. Residues in bold characters were confirmed by amino acid sequencing. The putative proteolytic motif is presented in a gray box. Pi: *Panulirus interruptus* [12], Pj: *Palinurus japonicus* [14], Alb: *Astacus leptodactylus* [36], CdM: *Cherax destructor*, Cp: *Cancer pagurus* and Cm: *Carcinus maenas* [14].

ception of two positions: base 435 was found to be G or C; and base 1038, A or G. Even when screening was performed using the short synthetic oligonucleotide probe with low stringent conditions, all the selected positive clones were inserts with this low level of polymorphism. Nonetheless, these two changes in the nucleotide sequence lead to a single change in the amino acid primary structure of the two subunits, thus showing that residue 131 could be a glutamine or a histidine.

The length recorded for the complete mature protein was 648 amino acids, corresponding to a mass of 73 570 Da and approaching the molecular mass as determined by SDS-gel electrophoresis (73–75 kDa). No putative carbohydrate attachment site was determined. In fact, N-type glycosylation is found only in *Panulirus* and *Astacus* hemocyanins [34], although some Asn-X-Ser/Thr sequences have been determined in other crustaceae [35]. Only one cysteine residue is shown, unlike *P. interruptus* (a) subunit, which presents three disulfide bridges, or *P. interruptus* (c) subunit with one disulfide bridge. In *P. vannamei*, the absence of these bridges may cause a certain flexibility in the molecule essential for cooperativity in oxygen binding.

Two overlapping N-terminal sequences were obtained. One was 6 amino acids longer than the other. The first sequence starts with phenylalanine in position 1 of the deduced mature protein sequence. The second protein sequence starts six residues later with the aspartic acid in position 7 of the deduced protein. The alignment of these sequences with other crustacean hemocyanins confirms the existence of subunits with truncated N-terminal portions (Fig. 3), possibly accounting, in part, for the difference in the observed molecular weights. Moreover, the presence of an Ala-X-Ala motive (position 4–6) inside the N-terminal sequence of the longest subunit explains the existence of the shorter subunit by a partial hydrolysis.

3.3. Sequence alignment

The mature protein sequence was aligned with sequences of the two subunits (a) and (c) found in *P. interruptus* [15,16]. The maximum percentage of identity is found when aligning *P. vannamei* and *P. interruptus* variant (c) hemocyanins (Fig. 4). In addition, the alignments showed the same location for several deletions specifically in the C-terminal domain of the two proteins. As expected, the second domain with the oxygen binding site was the most conserved part of the chain. The third domain had the largest number of replacements and

resembles more closely the *P. interruptus* (c) subunit, especially in the stretch 583–605. As suggested by Neuteboom et al. [15], hemocyanin (a) and (c) result from gene duplications that occur before the divergence of *Astacus* and *Panulirus*. Our results suggest that this duplication occurred after the appearance of Penaeidae, probably in the Paleozoic [38], since the percentage of identity of the *P. vannamei* subunit is more or less the same as that of the two *P. interruptus* subunits, (a) and (c). As such, *P. vannamei* hemocyanin may represent a primitive subunit [39].

3.4. Hemocyanin mRNA expression

Hybridization of total RNA from the hepatopancreas of *P. vannamei* with homologous cDNA probes showed a strong band approximately 2100 bp in length (Fig. 5, insert). This size corresponds to the longest isolated cDNA and fits well with the apparent sizes ranging from 1900 to 2300 bp as determined in *Callinectes sapidus* [40]. Our results show that hemocyanin is synthesized in the hepatopancreas confirming the results obtained in the crayfish using [³⁵S]methionine incorporation [41,42], in the blue crab using hybridization of hepatopancreas mRNA with an oligonucleotide based on the amino acid sequence encompassing the 'copper B' binding site [40], and recently in penaeids [43]. Moreover, we have shown that hemocyanin is synthesized as a preprotein in a very large amount (8% of the cDNA clones). In contrast, the frequency of trypsin or chymotrypsin (the two highly produced digestive enzymes) coding sequences in this cDNA library is only 0.5 and 0.6% respectively [44].

Variations in the amount of hemocyanin mRNA were studied during the moult cycle (Fig. 5). Hemocyanin mRNA amount increased largely from a minimum in stage A to a maximum in stages D1''–D1'''. This pattern has been observed in the expression of chymotrypsin [44], trypsin [27], and other proteins synthesized in the hepatopancreas. In all cases, minima are observed in stages D3–AB and maxima occurred during stages D1''–D1'''. These results corroborate the hypothesis of hemocyanin synthesis in hepatopancreatic cells starting in stage D0, with a coordinate release of cellular constituents under the stimulation by ecdysteroids during the premoult. Biphasic variations are seen for hemocyanin synthesis in *Astacus leptodactylus* [13] hepatopancreas in relation with ecdysteroid levels. The importance of ecdysteroids for the moulting process is emphasized by their stimulative effect on many

	10	20	30	40	50	60	70	80	
Pv	FQVASADVQQ	QKDVLYLLNK	IYGDIDQDGL	LATANSFDPV	GNLGSYSDDG	AAVQKLVDL	NDGKLLQKH	WFSLFNTRHR	
Pia	DALGTGNAQK	QDINHLDDK	IYEPTKYPDL	KEIAENFNPL	GDTSIYNHNG	AAVETLMKEL	NDHRLLEQRH	WYSLFNTRQR	
Pic	ADCQAG	DSADKLLAQK	QHDVNYLVYK	LYGDIRDDHL	KELGETFNPQ	GDLLLYHDNG	ASVNTLMADF	KDGRLLQKKH	WFSLFNTRQR

Domain 1

	90	100	110	120	130	140	150	160	170
Pv	NEALMLFDVL	IHCKDWASFV	GNAAYFRQKM	NEGEFVYALY	VAVIHSSLAE	QVVLPPLYEV	TPHLFTNSEV	IEEAYRAKQK	QTPGKFKSSF
Pia	KEALMLFAVL	NQCKEWCYFR	SNAAYFRERM	NEGEFVYALY	VSVIHSLGSD	GIVLPPLYEI	TPHMTNSEV	IDKAYSAMKT	QKQGTNFVSF
Pic	EEALMMHRVL	MNCKNWHAFV	SNAAYFRNTM	NEGEYLYALY	VSLIHSGLGE	GVVLPPLYEV	TPHMTNSEV	IHEAYKAQMT	NTPSKFESHF

Cu A

→

	180	190	*	*200	210	220	*	230	240	250	260
Pv	TGTTKKNPEQR	VAYFGEDIGL	NTHHVTWHME	FPFWNDAYG	HHLDRKGKGF	FWIHHQLTVR	FDAERLSNVL	DPVGELQWKN	PIVDGFAPHT		
Pia	TGTTKKNREQR	VAYFGEDIGM	NIHHVTWHMD	FPFWWEDSYG	YHLDRKGKGF	FWVHHQLTVR	FDFERLSNVL	DPVDELHWRD	IIREGFAPLT		
Pic	TGSKKNPEQH	VAYFGEDVGM	NTHHVLWHME	FPFWWEDSSG	RHLDRKGKGF	FWVHHQLTVR	YDAERLSNHL	DPVEELSWNK	AIDEGFAPHT		

Domain 2

	270	280	290	300	310	320	330	340	*	*350
Pv	TYKYGGQFPA	RPDNNKFDV	DDVARIRDMV	IVESRIRDAI	AHGYIVDSEG	KHIDISNEKG	IDILGDIIES	SLYSPNVQYY	GALHNTAHIV	
Pia	SYKYGGGEFPV	RPDNIHFEDV	DGVAHVHDL	ITESRIHEAI	DHGYITDSG	HTIDIRQPKG	IELLDGDIIES	SKYSSNVQYY	GSLHNTAHVM	
Pic	AYKYGGYFPS	RPDNNVHSDV	DGVARVRDMS	MTEDRIRDAI	AHGYIDALDG	HSIDIMNSHG	IEFLGDIIES	SGYSANPGFY	GSLHNTAHIM	

Cu B

	360	370	380	*	390	400	410	420	430	440
Pv	LGRQGDPHGK	FDLPPGVLEH	FETATRDPSF	FRLHKYMDNI	FKEHKDNLPP	YTKADLEFSG	VSVTELAUVG	ELETYFEDFE	YSLINAVDDA	
Pia	LGRQGDPHGK	FNLPPGVMEH	FETATRDPSF	FRLHKYMDNI	FKKHTDSFPP	YTHDNLEFSG	MVNGVAIDG	ELITFFDEFQ	YSLINAVDSG	
Pic	LGRQGDPTGK	FDLPPGVLEH	FETSTRDPSF	FRLHKYMDNI	FREHKDSLTP	YTRDELEFNG	VSIDSIAIEG	TLETFFENFE	YSLINAVDDT	

Domain 3

	450	460	470	480	490	500	510	520	530
Pv	EGIPDVEIST	YVPRLNHKEF	TFRIDV-ENG	AERLATVRIF	AWPHKDNNGI	EYTFDEGRWN	AIELDKFVWS	LKGGKTSIER	KSTESSVTVE
Pia	ENIEDVEINA	RVHRLNHKEF	TYKITMSNNND	GERLATFRIF	LCPIEDNNGI	TLTLDARWF	CIELDKFFQK	VPKGPETIER	SSKDSSVTVE
Pic	VDIADVEILT	YIERLNHKKF	SFLILVTNNNN	TEVLATVRIF	AWPLRDNNGI	EYSFNEGRWR	ALELDRFWVK	VKHGHQITR	QSTESSVTVE

Domain 4

	540	550	560	570	580	590	600	610
Pv	DVPSIHLDLFA	EAEAGGAG-----LA	KFESATGLPN	RFLLPKGNDR	GLEFDLVVAV	TDGDADSAVP	N-LHE-NTEYNH	YGSHG-VYPDK
Pia	DMPFSQSLKE	QADNAVNGGHDLDS	AYERSCGIPD	RMLLPKSKPE	GMEFNLYVAV	TDGDKDTEGH	NGGHDYGGTHAQ	CGVHGEAYPDN
Pic	DVPSLQTLID	RADAAISSGCALHLE	DYESALGLPN	RFLLPKGQAQ	GMEFNLYVAV	TDGRTDAALD	D-LHE-NTRKFIH	YGYDR-QYPDK

Domain 5

→

	620	630	640	
Pv	RPHGYPLDRK	VPDERVFEDL	PNFKHIQVKV	FNHGEHIH
Pia	RPLGYPLERR	IPDERVIDGV	SNIKHVVVKI	VHLEHHD
Pic	RPHGYPLDRR	VDERIFEAL	PNFKQRTVKL	YSHEGVDGG

Fig. 4. Alignment of arthropod hemocyanin amino acid sequences [37]. Amino acid sequences are shown for shrimp hemocyanin (Pv), for two of the three subunits of *Panulirus interruptus* (Pia and Pic) [15,16]. Gaps have been introduced in the sequences to facilitate alignments. The numbering above the alignments relates to the shrimp mature hemocyanin as used in Fig. 2. The two copper A and B binding sites are framed. Domain 1 and 2 extents are indicated by arrows.

protein expressions in different tissues, such as actin in crustacean muscle [45]. Actin, which is considered a reference protein in mammals, varies during the moult cycle and, therefore, cannot be used as a control for crustaceans.

Instead, R-cells may be a good candidate for the hemocyanin synthesis location because they display ultrastructural features for the export of metabolites to other organs through hemolymph [46–48]. Certain authors [49], however, consider that the reserve inclusion cells located in small hemal sinuses are responsible for the synthesis and the storage of hemocyanin. Such a hypothesis needs further substantiation since the number of these cells is very low.

In the future, the cDNA we described here will be used as a

probe to confirm the hepatopancreas cell type in which hemocyanin is synthesized.

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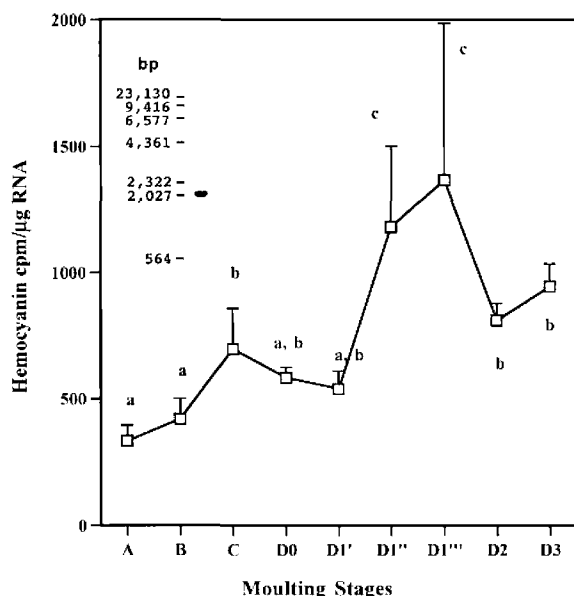


Fig. 5. Variation of the amount of hemocyanin messenger RNA during the intermoult cycle. RNA amounts were quantified by spectrophotometry. 1 μ l dots of total RNA extracted from individuals at different moulting stages were fixed on nylon membranes and hybridized as described in Section 2. The curve represents mean values of 2–6 determinations for each moulting stages. Standard errors of mean (S.E.M.) as well as Student analyses are indicated. The different letters correspond to significant values at 0.05 S. Insert: Northern blot of *Penaeus vannamei* total RNA.

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