



Purification of Hemocyanin from White Shrimp (*Penaeus vannamei* Boone) by Immobilized Metal Affinity Chromatography

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ABSTRACT. Hemocyanin (Hc) was isolated from white shrimp (*Penaeus vannamei* Boone) plasma by density gradient ultracentrifugation and immobilized metal affinity chromatography. Hc was contained in the subnatant high density fraction and was adsorbed by an iminodiacetic acid (Ni-IDA) column that bound Hc and apohemocyanin. Hc molecular weight was estimated by pore limiting electrophoresis as 400 kDa. It is composed of two subunits of approximately 75 and 82 kDa. This 400-kDa protein contained copper and was detected by antibodies raised against the purified subunits. Isoelectric points of the affinity purified native protein were 4.8 and 4.9. Presence of covalently bound carbohydrates in both subunits was detected by biotinylated lectins and avidin-horseradish peroxidase. COMP BIOCHEM PHYSIOL 117B;2:203–208, 1997. © 1997 Elsevier Science Inc.

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INTRODUCTION

Hemocyanin (Hc) is a copper-containing protein involved in the transport of oxygen (40). It is present at high concentrations in crustaceans, comprising 90–95% of the total plasma protein in *Palaemon elegans* and *Penaeus vannamei* (7,9,37). Hc oxygen affinity is affected by changes in pH and temperature and also by concentrations of lactate, calcium and sodium, providing in this form an adaptation mechanism for oxygen transport under stress conditions (4,5). Besides transporting oxygen, Hc is an important storage protein, an osmoregulator and is involved in the transport of metals and aminoacids (47).

Crustacean Hc hexameric proteins can associate to form aggregates from 12 (2×6) up to 48 (8×6) subunits, resulting in molecular masses from 450 to 3900 kDa (14). The most common Hc arrangement for decapods is hexameric or dodecameric (1×6 and 2×6) (8). Hc has been the subject of many reports due to its physiological importance and biochemical characteristics. The isolation procedures used are mainly ultracentrifugation and/or gel filtration chromatography (26,32,33). The heterologous crustacean Hc subunits have been classified according to their electro-

phoretic mobility, participation in the native protein assembly or immunological characteristics in three types: alpha, beta and gamma (24). The subunit heterogeneity has been related with seasonal and/or sex variations (1,6,14,18). In addition, arthropod hemocyanins are generally glycosylated, containing 2–9% (w/w) of carbohydrate with very variable composition (27,36). The variability in carbohydrate composition may also contribute to the microheterogeneity of the subunits (27).

The isolation of other proteins present in lower concentration than Hc from crustacean plasma is often complicated by the presence of Hc as a major contaminant protein. Furthermore, there is little information about white shrimp (*P. vannamei*) plasma proteins, and this is one of the most important cultured species. Understanding its biochemical components is an essential step toward optimizing the conditions for cultivation. Hc from white shrimp was isolated and characterized to understand more about the chemistry of crustacean plasma proteins, to obtain a putative marker for diagnostic purposes and to find a method to remove Hc from shrimp plasma for other protein studies. A two-step purification procedure was used: ultracentrifugation and immobilized metal ion affinity chromatography (IMAC). IMAC has been used to isolate proteins, peptides and nucleic acids and for cell separation [for a review on IMAC and its principles, see (48)]. Proteins from different origins have been isolated by IMAC, including vertebrates (44), insects (31,49), fish (45) and recombinant fusion proteins

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(20), but to our knowledge, this is the first report on the use of IMAC for the purification of crustacean Hc.

MATERIALS AND METHODS

Chemicals

Reagents for polyacrylamide gels were from Bio-Rad (Richmond, CA), protease inhibitors were from Sigma Chemical (St Louis, MO), biotinylated lectins from Pierce (Rockford, IL) and Phast Gels and buffer strips from Pharmacia LKB Biotechnology (Uppsala, Sweden). Sepharose 6B-IDA was a kind gift from Dr. J. Porath (University of Arizona, Tucson, AZ). All other reagents were analytical grade or superior.

Hemolymph Extraction

Hemolymph was extracted from apparently healthy juvenile white shrimp (*P. vannamei*) obtained from aquaculture farms in Bahia Kino, Sonora, Mexico, or from The Center for Biological Research (La Paz, BCS, Mexico). Hemolymph was collected separately in anticoagulant solution (41), containing protease inhibitors (50), and the hemocytes removed (41).

Hemocyanin Purification

Two steps were used for the isolation: a density gradient ultracentrifugation and an IMAC.

ULTRACENTRIFUGATION. Density gradient ultracentrifugation was performed as described (50). The density gradient was separated from the top to the bottom in four fractions: a clear fraction above the lipoprotein (fraction I), the yellowish lipoprotein fraction previously characterized (fraction II) (50), a clear fraction below the lipoprotein (fraction III) and a high density blue bottom fraction (fraction IV). Fraction IV was desalted and prepared for the next step by passing it through a PD10 column (Bio-Rad) and eluted with buffer A (0.5 M NaCl, 0.05 M Tris, pH 7.5).

IMAC. A 1 × 15-cm column with a 3-ml matrix was used. It consisted of Sepharose 6B with bound iminodiacetic acid (IDA) groups, which in turn were coordinated with nickel ions. The Ni²⁺ ions were immobilized by incubating the matrix for 1 hr with 0.1 M Ni₂SO₄ and rinsed with 6 volumes of water. After the column was equilibrated with buffer A, 4.5 mg of fraction IV were loaded and rinsed extensively with buffer A to remove the unbound proteins. Proteins adsorbed by Ni-IDA matrix were eluted by using a 7.5–4 pH linear gradient made with 0.5 M sodium acetate, 0.5 M NaCl solutions with the two different pHs in a gradient former (GIBCO BRL, Gaithersburg, MD). One chamber contained 15 ml of the above-mentioned solution at pH 7.5 and the other the same solution but at pH 4. The former gradient was directly connected to the chromatographic

equipment with a flow of 1 ml/min. Two-milliliter fractions were collected separately. The column was finally washed with 40% ethylene glycol. Protein chromatography elution was monitored by absorbance at 280 nm, and protein concentration in the fractions was determined using bovine serum albumin as standard (3).

Determination of Cu Concentration

Aliquots from each purification step were digested in an MDS-81D microwave digestion system (CEM, Mathews, NC) and quantified by flame atomic absorption (SpectrAA-20, Varian, Mulgrave, Australia) (13).

PAGE and SDS-PAGE

After each purification step, aliquots of protein containing fractions were dialyzed against 10 mM phosphate, 15 mM NaCl, pH 7.5 at 4°C for 1 hr and loaded on to a polyacrylamide gel. Fractions from ultracentrifugation and column chromatography were analyzed in 6–18% discontinuous PAGE and SDS-PAGE reducing slab gels (19) and stained with 0.25% Coomassie Brilliant Blue R250. In addition, in native gels, Hc was identified by staining for the presence of Cu with 0.1% dianisidine (23). Electrophoretically separated polypeptides were also blotted to nitrocellulose membranes (NC) in a Trans-Blot Semi dry (Bio-Rad) at 20 V, 45 min with 48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2.

Carbohydrate Detection

Protein glycosylation was detected in native and denaturing gels using PA/S staining (35). The type of carbohydrate in the subunits was investigated in NC Hc blots probed with biotinylated lectins (10 µg/ml of lectin in TBS [500 mM NaCl, 20 mM Tris, pH 7.5]) for 2 hr. The lectins tested were concanavaline A (ConA), *Ulex europaeus* I agglutinin (UEA I), wheat germ agglutinin, soy bean agglutinin (SBA), *Dolichus biflorus* agglutinin (DBA), peanut agglutinin (PNA) and *Ricinus communis* (RCA). After incubation with the lectins, the blots were washed three times with TBST, incubated with avidin-conjugated horseradish peroxidase for 1 hr, washed again three times with TBST and peroxidase activity detected with 4-chloro-1-naphthol.

Isoelectric Focusing

Isoelectric focusing was carried out using homogenous native gels in the Phast System Separation (Pharmacia, LKB Biotechnology). A pH gradient from 3 to 9 was generated. Affinity-purified Hc was dialyzed for 24 hr against distilled deionized water before loading on to the gel. Protein standards had a pI range from 3.6 to 9.3 (Sigma Chemical Co.).

The gel was silver stained according to the Phast System protocol (Pharmacia).

Antibody Preparation, Purification and Characterization

Polyclonal antibodies were prepared against the 75- and 82-kDa subunits, as recommended by Jurd and Bog-Hansen (16). The bands were excised from preparative SDS-PAGE, injected into rabbits and tested as described (39). The antibodies were designated as anti-75 and anti-82, respectively. Western blots were done as described (39).

RESULTS AND DISCUSSION

Purification of Hc

ULTRACENTRIFUGATION. The highest density blue fraction (fraction IV) corresponded to 87.7% of the total plasma protein. Hc represents 80–95% of the hemolymph protein from *P. vannamei* (7), with a blue color after oxygenation (30). Therefore, this fraction was considered as rich in Hc and prepared for the next step.

METAL AFFINITY CHROMATOGRAPHY. This method is based on the separation of proteins by the coordination between imidazole groups exposed in the protein surface and the ions of an immobilized metal in the solid matrix (29) and has been demonstrated to be a good option for isolation of proteins (19,31,45,48). Thus, we tried white shrimp Hc purification by applying 4.5 mg of the blue fraction IV to a 3-ml Ni-IDA column. After extensive column washing, two peaks were clearly separated: peak A corresponding to the unbound proteins and representing 2.7% and peak B, the protein eluted along the pH gradient (7.5–4) and containing 87.3% of the protein loaded on to the column (Fig. 1). Two proteins were bound to Ni-IDA and identified as Hc and apohemocyanin (see below). Both were eluted at the same time, and despite the use of different conditions for elution, it was not possible to separate Hc and apoHc. Therefore, it appears that copper binding to the apoprotein does not change the exposed histidine residues, which are responsible for the interaction with the Ni ions. A small fraction of Hc did not bind to the IMAC column (Fig. 1, peak A) and was independent of the amount of protein loaded onto the column; therefore, these molecules appear to contain a slightly different composition or conformation at presently not understood.

Electrophoretic Analysis

A very intense band of 400 kDa and a weak band of 90 kDa were detected by native pore limiting PAGE (Fig. 2); the first one coincides with reports of 471 kDa for Hc from *P. monodon* and *P. setiferus* (4,12). This is also close to the size of a hexamer. Two bands of 82 and 75 kDa and a protein smear were present in the fractions eluted from Ni-IDA with the pH gradient and corresponding to peak B (Figs 1

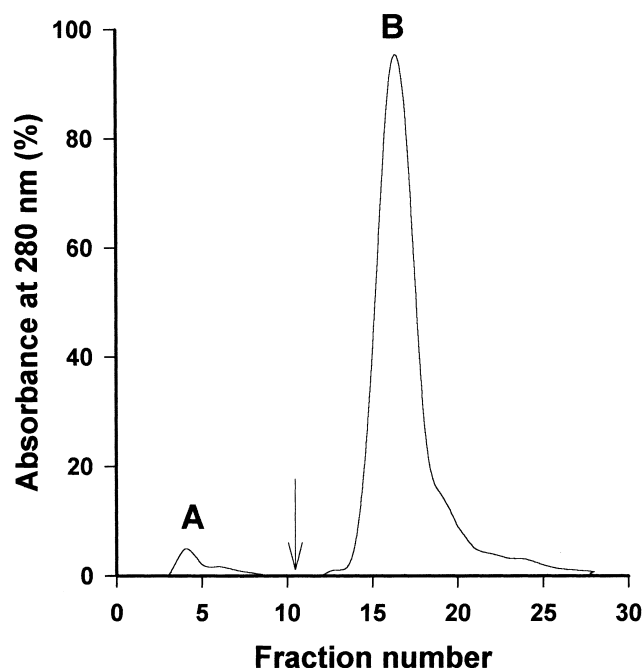


FIG. 1. Purification of Hc by metal affinity chromatography on Ni-IDA-Sepharose. Fractionation of 4.5 mg of fraction IV from the density gradient ultracentrifugation. Peak A, protein not adsorbed to the matrix. Peak B, protein eluted with the pH gradient. Arrow indicates point of application of pH gradient. Hc elution started approximately at pH 6.

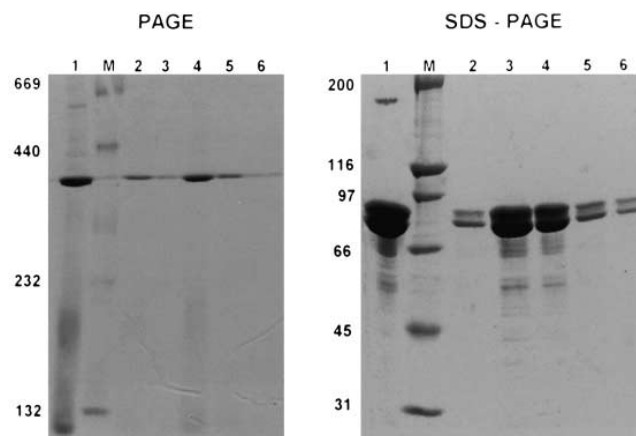


FIG. 2. Electrophoretic analysis of the fractions from the two steps purification. Native (PAGE) and denaturing and reducing (SDS-PAGE) 6–18% gradient slab gels. Lanes on both panels contain the same samples. Lane M, molecular weight markers, lanes 1, fraction IV from the density gradient ultracentrifugation step; lanes 2, sample not adsorbed by the column, (peak A in Fig. 1); lanes 3–6, fractions (min) 13–16 eluted with the pH gradient and corresponding to peak B in Fig. 1.

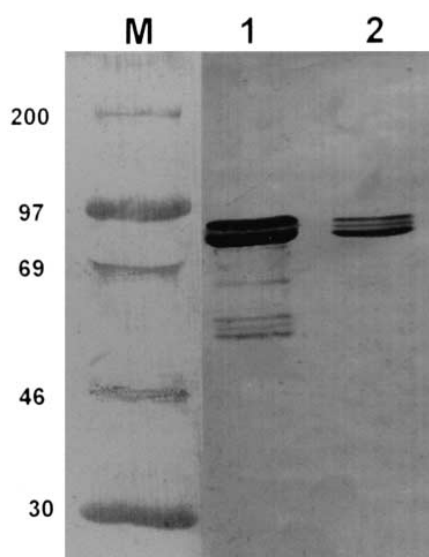


FIG. 3. Recognition of Hc subunits by the polyclonal anti-75 kDa antibodies. Similar patterns were obtained with anti-75 and anti-82 antibodies. Lane M, molecular weight markers; lane 1, 1 μ g of Hc; and lane 2, 0.1 μ g.

and 2). Subunits of 82 and 77 kDa were reported for *P. setiferus* (4) and three Hc subunits (85, 77 and 70 kDa) in *P. monodon* (12). Recently, Khayat *et al.* (17) reported *P. semisulcatus* Hc polypeptides of 64 and 73 kDa that are specifically synthesized by the hepatopancreas. The 400-kDa band in PAGE was stained by dianisidine, indicating the presence of Cu as reported by (8,23). The 90-kDa band also contained the two Hc subunits but did not stain for Cu presence; therefore, it appears to correspond to apoHc. Furthermore, Hc as well as the 75- and 82-kDa polypeptides were recognized by both anti-75 and anti-82 polyclonal antibodies (Fig. 3). A 600-kDa band was also present in fraction IV from ultracentrifugation, but it was not detected in Coomassie Blue or silver-stained gels of metal affinity purified Hc (Fig. 2, lane 1) and was not recognized by either antibody (Fig. 3). Therefore, this band does not appear to be related to Hc. The identity of this band has not been elucidated yet but may be related to proteins involved in coagulation and/or the defense system, because these kinds of proteins are present in shrimp plasma (42,43).

Purified native white shrimp Hc presented two bands with very close pIs of 4.8 and 4.9. The protein with pI of 4.9 was less abundant and probably corresponds to apohemocyanin. In *Palunirus japonicus*, the pIs reported (21) for the four subunits are 5.38 (79 kDa), 5.06 (84 kDa), 5.11 (88 kDa) and 5.10 (90 kDa). A recent report comparing Hc pIs from decapoda included only *P. indicus*, from the family Penaeidae with four bands in the electrophoregrams and with pIs for the main bands of 4.2 and 4.25 (28).

In different preparations of Hc, smaller bands were detected in the gels, but only the bands of 75 and 82 kDa were consistently present; therefore, we believe they are

degradation products of the two subunits. The analysis of the Hc subunits, despite the abundance of the protein, defining number and sizes has been hampered by anomalous migration on gels. In addition, smaller bands seem to result from polypeptides breakdown in susceptible sites (15,22, 25). In other cases, the relative abundance of the subunits appears to be dependent on season (10).

The relative abundance of the two Hc subunits in Coomassie as well as in silver-stained gels seemed to be slightly different. An attempt to estimate their proportions was done by scanning a Coomassie-stained gel and quantifying each band. A 1:1.5 ratio between the 82- and 75-kDa subunits was obtained, and this is close to the 1:2.6 ratio for the corresponding subunits from *P. setiferus* (12).

Cu:Protein Ratio

Copper and protein concentrations were determined in whole shrimp, plasma and fraction IV. Values of 0.0157, 0.4350 and 1.3050 μ g Cu/mg protein, respectively were obtained, indicating increasing concentrations of Cu. In fraction IV, this corresponds to approximately 0.13% Cu, and this fraction contains at least Hc and apoHc and the 600-kDa band. This is compatible with reports of 0.15–0.2% of Cu in Hc (11). Several studies have shown that the ratio between copper and protein in Hc is in this range, regardless of starvation (34) or stressed conditions (2); [for a review, see (11)].

Glycosylation of Hc

By using PA/S staining and biotinylated lectins, we detected carbohydrates in the native Hc and denatured subunits. A positive reaction was obtained with ConA, UEA, DBA and PNA. These lectins specifically recognize α -D-mannose and α -D-glucose, α -L-fucose, α -D-N acetyl galactosamine and β -D-gal(1-3)D-N-acetyl-galactosamine, respectively (Table 1). Therefore, this means that Hc is a glycosylated protein. The presence of mannose and glucose residues was reported for subunit "a" (94 kDa) and "c" (80 kDa) from *Panulirus interruptus*; these subunits differ only in the type of glycosylation and percent of carbohydrates (27,46). *Rapana thomasi* Hc, in addition to mannose, ga-

TABLE 1. Interaction of Hc from *P. vannamei* with biotinylated lectins

Lectin	Recognized carbohydrate	Reaction
ConA	α -D-mannose, α -D-glucose	Positive
UEA I	α -L-fucose	Positive
WGA	(D-N-acetyl-galactosamine) ₂	Negative
SBA	D-N-acetyl galactosamine	Negative
DBA	α -D-N-acetyl galactosamine	Positive
PNA	β -D-gal-(1-3)D-acetyl galactosamine	Positive
RCA	β -D-galactosamine	Negative

lactose and fucose, contains three other carbohydrates, including xylose, 3-O-methylgalactosamine and N-acetylglucosamine (36). In the crayfish *Astacus leptodactylus* Hc, only one subunit is glycosylated, containing six N-linked mannose rich and N-acetylglucosamine (38). No differences between sexes were detected in Hc from juvenile shrimp concerning size, binding to the column, interaction with lectins or binding of the antibodies.

In summary, the two steps used to purify Hc, namely ultracentrifugation and IMAC, proved to be very useful. Hc was adsorbed by the Ni-IDA column and eluted specifically with the pH gradient. Although a small amount of Hc was not bound by the metal affinity column, this is probably due to slight differences in conformation and/or composition of the polypeptides, because the gel capacity was not exceeded. Our Hc purification procedure can be used to obtain pure Hc for latter studies or to remove Hc from samples desired for purification of other less abundant or rare proteins. Hc from the white shrimp has an apparent molecular mass of 400 kDa and is composed by two glycosylated polypeptides of 75 and 82 kDa. Antibodies raised against each of the gel purified subunits recognized both subunits. We are currently studying the individual Hc subunits and conducting experiments with the anti-Hc antibodies to follow the breakdown of the 75- and 82-kDa subunits.

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