PURIFICATION OF HEMOCYANIN FROM MARINE GASTROPOD *RAPANA THOMASIANA* USING AMMONIUM SULFATE PRECIPITATION METHOD

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ABSTRACT
The aim of this study was to develop an efficient and simple method for isolation of preparative amounts of pure hemocyanin (Hc) from the hemolymph of the marine gastropod Rapana thomaisana. The methods that are usually used, as ultracentrifugation and column chromatography, are expensive and impractical for the large-scale production of Hc. For the ammonium sulfate precipitation method, the concentrated hemolymph was twice precipitated by 38% saturation with crystalline ammonium sulfate. *Rapana thomaisana* hemocyanin (RtH) was isolated with good yield and high purity, as assessed by gel chromatography, SDS-PAGE, transmission electron microscopy and absorption spectroscopy. This suggests that the ammonium sulfate precipitation is an efficient and useful purification method, suitable for a large-scale RtH preparation.

Keywords: *Rapana thomaisana*, gastropod, hemocyanin, ammonium sulfate

Introduction
Hemocyanins (Hcs) are blue copper-containing respiratory proteins freely dissolved in the hemolymph of many arthropods and molluscs (13, 18). Molluscan Hcs, have a hollow cylindrical quaternary structure, ca. 35 nm in diameter, and are constructed from either ten (cephalopod Hcs) or twenty (gastropod Hcs) subunits. The subunit of gastropod Hcs is an approximately 400 kDa glycosylated polypeptide folded into eight globular substructures, the so-called “functional units” (FUs), which are termed FU-a to FU-h (from the N- to the C-terminus). Each FU has a molecular mass of approximately 50 kDa and contains one binuclear copper active site, capable of reversibly binding one dioxygen molecule. The eight FUs in each subunit are connected by short linker region of the polypeptide chain.

Molluscan Hcs have been intensively studied for their structure, function and for immunological and clinical applications. The keyhole limpet hemocyanin (KLH) isolated from the hemolymph of the marine gastropod *Megathura crenulata* is the most widely used as an adjuvant and a carrier for vaccines and antigens (4, 5).

*Rapana thomaisana* (prosobranch gastropod) is a marine snail, originally found in the China Sea and along the coasts of Japan. In 1947 this species was transferred to the West coast of the Black Sea, where it adapted. New Hc has been isolated and purified from the hemolymph of this marine gastropod using ultracentrifugation and column chromatography (6). *Rapana thomaisana* hemocyanin (RtH) has been structurally well characterized (2, 3, 6, 7, 8, 9, 10, 12, 14, 15, 16). Recently, we demonstrated the high immunogenicity of RtH as a model antigen and also its properties as a strong protein carrier for viral peptides from Influenza hemagglutinin (17). The results obtained suggested a potential role of RtH as an acceptable compound needed for adjuvanicity for standard vaccines.

The availability of *Rapana thomaisana* inexhaustible natural source such as the Black Sea, as well as the possibilities for an application of RtH in the laboratory and clinical practice, prompted us to create an effective scheme for its preparation. Ultracentrifugation and column chromatography are expensive and impractical for the large-scale production of Hc. Therefore, the aim of this study was to develop an efficient and simple method for large-scale production of RtH with high purity and yield rates.

Materials and Methods
Collection of hemolymph from marine snails *Rapana thomaisana*
Living marine snails *Rapana thomaisana* were caught near the Bulgarian coast of Black Sea (Varna) and stored in sea water. The hemolymph was collected by bleeding through several diagonal slits made on the foot of the mollusc and filtered through gauze. Phenylmethanesulphonyl fluoride (PMSF) (1mM final concentration) was added to the crude material to avoid possible proteolysis of the hemolymph. Hemocytes and other cells were removed by centrifugation at 5000 g for 30 min at 4°C. The hemolymph was concentrated by the ultrafiltration system Tangential Ultrafiltration ProVario-3 (Pall-Filtron, Dreieich, Germany). Sucrose was added as a cryoprotector to a final concentration of 20% (w/v) and the hemolymph stored at -20°C until use.

Isolation of *Rapana thomaisana* hemocyanin
The native RtH was purified from the concentrated hemolymph by precipitation at 38% saturation with crystalline ammonium sulfate, and then twice precipitated by 38% saturation with crystalline ammonium sulfate. RtH was isolated with good yield and high purity, as assessed by gel chromatography, SDS-PAGE, transmission electron microscopy and absorption spectroscopy. This suggests that the ammonium sulfate precipitation is an efficient and useful purification method, suitable for a large-scale RtH preparation.
The RtH pellet was suspended in 50 mM Tris/HCl, pH 7.4 and dialyzed at 4°C against the same buffer, in order to remove the ammonium sulfate. Finally, the material was dialyzed against “stabilizing buffer” (50 mM Tris/HCl, 150 mM NaCl, 5 mM CaCl₂, 5 mM MgCl₂, pH 7.4). The RtH solution was clarified by centrifugation for 20 min at 3 000 x g. The isolated Hc, with a concentration of about 20 mg/ml, was stored at -20°C in presence of 20% (w/v) sucrose. Alternatively, Hc solution was sterilized by filtration through a microporous membrane, and stored at 4°C.

**Gel chromatography**

Gel filtration chromatography of isolated native RtH was performed on a column Sepharose 4B (90 x 2.0 cm), equilibrated and eluted with buffer 50 mM Tris/HCl, pH 7.4, containing 5 mM CaCl₂ and 5 mM MgCl₂.

**Absorption spectroscopy**

Absorption spectra of RtH were recorded with a spectrophotometer Shimadzu model 3000. Protein concentration was determined spectrophotometrically using the absorption coefficient A₂₇₈₀.₁% = 1.36 mg⁻¹ ml⁻¹.

**Transmission electron microscopy (TEM)**

The sample of native RtH was adsorbed to a glow-discharged Pololtem/carbon coated support film, washed with distilled water to remove the buffer salts and negatively stained with 5% (w/v) ammonium molybdate containing 1% trehalose at pH 7.0. Sample was viewed in a Philips CM 10 transmission electron microscope at 60 kV acceleration voltage and an instrumental magnification of 52 000.

**SDS gel electrophoresis**

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 7.5% polyacrylamide gel, according to Laemmli (11). The proteins used as standards for molecular mass determination were: myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), ovotransferrin (78 kDa), glutamate dehydrogenase (56 kDa), ovalbumin (42.7 kDa).

**Results and Discussion**

The respiratory proteins, Hcs, occur freely dissolved in the hemolymph of mollusks. Native Hc can be obtained by preparative ultracentrifugation of extracted hemolymph. In our pervious investigations, we have isolated RtH from freshly obtained hemolymph from marine snails *Rapana thomasiana* by pelleting in an ultracentrifuge (Beckman L-M-80, rotor Ti 45) at 180 000 x g for 4 hours at 4°C. However, in an actual large-scale commercial process it will be better to apply a method with enhanced productivity and lower cost price. The salting-out procedure has been usually employed for protein separation and purification. Among several types of salts, ammonium sulfate is the most widely used chemical because it has high solubility and is relatively inexpensive. In this study, alternative method for preparative isolation of RtH from the hemolymph by ammonium sulfate precipitation was developed.

Marine snails (size 9 x 7 cm) weighting approximately 100-120 g yielded approximately 15 ml hemolymph with Hc concentration approximately 5 mg/ml. Initially, diluted hemolymph (volume 1500 ml) was concentrated by means of an ultrafiltration system. Native RtH was purified from the concentrated hemolymph by precipitation at 38% saturation with crystalline ammonium sulfate for 12 h at 4°C and subsequent centrifugation for 1 h at 10 000 x g, at 4°C. The resultant pellet consisted primarily of high-molecular-weight RtH. The clear supernatant was discarded. Precipitation and concentration procedures were repeated twice. After removing of the ammonium sulfate by dialysis, the material was concentrated and finally dialyzed against the stabilizing buffer. The isolated Hc, with a concentration of about 20 mg/ml, can be stored at -20°C in presence of 20% (w/v) sucrose as cryoprotector. Alternatively, the Hc solution can be stored at 4°C after sterilization by filtration through a microporous membrane (0.22 μm pore size). Applying the described method approximately 5 g of pure Hc was obtained from 10 kg of marine snails.

The evaluation of purity and chemical characterization of the RtH molecule by means of SDS- and native-PAGE and N-terminal sequence analysis was described in details in our preceding works (6, 8). The isolated by ammonium sulfate precipitation RtH was characterized by gel chromatography, SDS-PAGE, TEM and absorption spectroscopy and the results were compared with the data obtained for RtH isolated according to (6, 8).

![Fig. 1. Gel filtration of RtH, isolated from the hemolymph of marine gastropod *Rapana thomasiana* by precipitation with ammonium sulfate (○●) and RtH, isolated by ultracentrifugation of the hemolymph (△▼), on a Sepharose 4B column (90 x 2.0 cm), equilibrated and eluted with 50 mM Tris/HCl, pH 7.4, containing 5 mM CaCl₂ and 5 mM MgCl₂. Insert: SDS-PAGE on 7.5% running gel of RtH under reducing conditions. Line 1: RtH isolated by precipitation of the hemolymph with ammonium sulfate; line 2: protein markers with the following molecular masses (from the top): myosin, 200 kDa; β-galactosidase, 116.3 kDa; phosphorylase b, 97.4 kDa; ovotransferrin, 78 kDa; glutamate dehydrogenase, 56 kDa; ovalbumin 42.7 kDa](image)
The purification of Hc from the hemolymph of marine gastropod *Rapana thomasiana* yielded pure protein preparation as assessed by gel filtration chromatography on a Sepharose 4B column and SDS-PAGE (Fig. 1). Both Rth samples were eluted as single and symmetrical peaks, indicating that the proteins were homogeneous in size. SDS-PAGE disclosed similar to other Rth preparations banding profile.

An electron microscope preparation of purified Rth molecules negatively stained with 5% (w/v) ammonium molybdate is shown on Fig. 2. It is evident that the isolated material is homogeneous. Only didecameric cylindrical aggregates of Rth, typical for gastropodan Hcs, were identified in the micrograph. No dissociated material or tubular structures were observed.

![Fig. 2. Electron micrograph of specimen prepared from isolated native Rth in buffer 50 mM Tris/HCl, pH 7.4, containing 5 mM CaCl₂ and 5 mM MgCl₂. The specimen was negatively stained with 5% ammonium molybdate, containing 1% trehalose. The scale bar indicates 100 nm](image)

The absorption spectrum of Rth shows characteristic bands at 280 and 345 nm, corresponding to the aromatic residues and Cu²⁺–O₂⁻–Cu₆⁺ complex at the active site, respectively (Fig. 3). The Rth was isolated with preserved active sites, as indicated by the absorbance ratio $A_{345}/A_{280} = 0.25$ typical for fully oxygenated Hc (1).

![Fig. 3. Absorption spectrum of isolated Rth in buffer 50 mM Tris/HCl, pH 7.4, containing 5 mM CaCl₂ and 5 mM MgCl₂](image)

**Conclusions**

The ammonium sulfate precipitation of hemolymph, collected from marine snails *Rapana thomasiana*, produces Hc with high purity and yield and is suitable for a large-scale Rth preparation.

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**REFERENCES**