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Biochemical Studies on *Siphonaria* (Gastropoda: Pulmonata) From the Karachi Coast of North Arabian Sea

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Abstract.- The present study showed the occurrence of three species of *Siphonaria*, namely, *S. ashgar, S, belcheri* and *S. kurracheensis* on the rocky coast of Mubarak village, Karachi, Pakistan. Keeping in view the biomedical interest in the high molecular mass hemocyanin of the marine mollusc, the study provides for the first time, the basic knowledge about the presence and chemical composition of oxygen transport protein hemocyanin in *Siphonaria* species. The tissue homogenates of *S. ashgar, S. belcheri* and *S. kurracheensis* when subjected to polyacrylmaide gel electrophoresis under dissociating conditions showed one major band (*ca.* 400kDa) which corresponds to two hemocyanin subunits (named as *Siphonaria* subunit S1 and S2). Under reducing conditions this band splits into three major bands, one major approximately 400kDa corresponding to the subunit S1, while S2 splits to produce two i.e. a 280kDa fragment S2.1 (corresponding to six-FUs) and the other 100-120kDa fragment S2.2 (corresponding to two-FUs). The size exclusion and ion-exchange chromatography results further revealed two specific major peaks corresponding to the two hemocyanin subunits. We conclude that the subunit composition of hemocyanin from *Siphonaria* resembles to the hemocyanin of other reported gastropods and thus could also be subjected for further immunological investigations for their possible biomedical application.

Key Words: Hemocyanin, *Siphonaria* species, Karachi Coast, Arabian Sea, pulmonate gastropods.

INTRODUCTION

The distribution and abundance of gastropod fauna on the rocky shores of Pakistan has been reported by various workers (Khan and Dastagir, 1971; Ahmed *et al*., 1982; Barkati and Burney, 1991; Moazzam and Ahmed, 1994; Ahmed and Hameed, 1999; Nasreen *et al*., 2000) but none have reported the presence of *Siphonaria* in their collections. This could be due to their conchological resemblance with patellid limpets. However, earlier reported species of *Siphonaria* from Pakistan include *S. kurracheensis*, *S. javanica sipho* and *S. lecanium* (Melvill and Standen, 1901).

The Siphonariidae (Gastropoda: Pulmonata) is a diverse family with over 60 species occurring globally (Hubendick, 1946). The majority of the species are found in the Indo-Pacific region especially in the southern hemisphere (Hubendick, 1946). Hemocyanin of the marine mollusc has been reported to possess remarkable immunostimulatory properties in experimental animals and man and has been scientifically studied for this purpose for the last 40 years (Harris and Markl, 1999). Since the genus *Siphonaria* belongs to the Phylum Mollusca, the presence of extracellular, blue, copper containing, glycoprotein "hemocyanin" which occurs freely dissolved in the hemolymph is anticipated. Although hemocyanin is found in the hemolymph, however, various parts in the animal body have been identified as the site of hemocyanin biosynthesis, such as the branchial glands in the cephalopods *Octopus dofleini*, *O. vulgaris* and *Eledone moschata* (Messenger *et al.*, 1974; Muzii, 1981; Lang, 1988), the ovoid cells of the branchial heart in *Sepia* sp. (Beuerlein *et al*., 1998), and the hepatopancreas in *Nautilus pompilius* (Ruth *et al.*, 1988, 1996). Ruth *et al.* (2000) reported that the four pericardial appendages of *Nautilus* are also involved in hemocyanin metabolism. In the pulmonate gastropods *Lymnaea stagnalis*, *Helix aspersa* and *Arion hortensis*, the class to which *Siphonaria* also belongs, "pore cells" (recently renamed "rhogocytes", for review, see Haszprunar

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1996) scattered among the connective tissue have been proposed as candidates for the site of hemocyanin biosynthesis (Sminia and Boer, 1973; Skelding and Newell, 1975; Sminia and Vlugt van Daalen, 1977). Although the characterization and organization of hemocyanin has been reported in number of molluscan species (Gebauer *et al*., 1994; Swerdlow *et al*., 1996; Harris and Markl, 1999; Markl *et al*., 2001; Lieb *et al*., 2010) but no information is available on the presence or characteristics of hemocyanin from *Siphonaria* species. Therefore, in the present study this protein was targeted to serve as internal protein marker beside other unknown proteins as point of differentiation. The data includes the protein finger-printing by polyacrylamide gel electrophoresis (native and SDS-PAGE) and size exclusion (SEC) and ion exchange (IEX) - Fast Protein Liquid Chromatography (FPLC). The other objective of the study was to identify the species of *Siphonaria* found on the coast of Karachi.

MATERIALS AND METHODS

Specimen collection and identification

About twenty specimens of each *Siphonaria* species were collected randomly from the rocky coast of Mubarak Village, Karachi (Fig. 1). The specimens were brought to the laboratory and washed with fresh water. The *Siphonaria* species were identified based on the shell morphology as described in the shell guide by Bosch *et al.* (1995). The three species of *Siphonaria* are found at three different tidal levels, *S. ashgar* at tidal height of about 3 meter, *S. belcheri* at tidal height of about 1-2 m and *S. kurracheensis* approximately at the tidal height of about 0.5 meter (Bano *et al*., 2011). The tides of the Karachi Harbour are semi diurnal type with average tidal height of 3.0 m (Quraishee, 1975).

Biochemical characterization

For the estimation of total protein, carbohydrate and lipid in the three species of *Siphonaria*, the whole body tissue was removed from 10 fresh specimens and pooled together, washed with 100mM phosphate buffer (pH7.0) and then one gram of the wet tissue taken for analysis. The tissue homogenate was prepared in 10 ml (1:10 w/v) of 100mM phosphate buffer (pH 7.0) using Polytron (PT-MT-2100, Kinematica Switzerland). Protein was estimated by colorimetric method as described by Lowry *et al.* (1951). Carbohydrate was estimated by the Phenol-Sulphuric acid (Dubois *et al.*, 1956) and total lipids by Sulphovanillin method (Barnes and Blackstock, 1973). For protein finger printing approximately one gram of tissue was washed and homogenized (Polytron PT-MT-2100, Kinematica, Switzerland) in 10 ml (1:10 w/v) of 100mM phosphate buffer (pH 7.0). The homogenates prepared were centrifuged twice at 15 000 rpm (Biofuge PrimoR, Heraeus Japan) for 30mins and the fat cap was removed before using the supernatant for subsequent processes. All steps were carried out on ice and/or 4°C.

Native and SDS-PAGE

For the determination of the tentative molecular mass and the protein finger prints, the protein preparations were subjected to 6% Native and 8% dissociating and/or dissociating-denaturing polyacrylamide gel electrophoresis (Mini PROTEAN 3 BioRad, UK) as described by Ali *et al.* (1995, 2007). Protein calibration marker (Emresco, USA) was run for the SDS-PAGE (Laemmli, 1970). The gels were stained with Coomassie Brilliant Blue R-250 (Fluka, EU). Image processing was performed using Adobe Photoshop CS 2.

SEC/IEX fast protein liquid chromatography (SEC/IEX - FPLC)

For all chromatographic steps, protein preparations were dialyzed in their respective buffer in the regenerated cellulose dialysis membrane (cut-off 12-14 kDa; Spectrum Labs, Europe) over night at room temperature (25°C). The samples were filtered through 0.22µ syringe filter (Millipore, USA) and directly subjected to fast protein liquid chromatography (AKTA-Design, Amersham Biosciences, UK) using TSK-2000SW column (300x7.5 mm; 10µ; Tosoh Bioscience, USA) equilibrated and eluted with (*a*) 20mM Imidazole-HCl buffer, pH6.5 containing 1mM $CaCl_2$ and 0.5mM $MgCl_2$, and (*b*) 100mM Phosphate buffer, pH7.0, (*c*) 50mM Tris-HCl buffer, pH8.5. The flow

rate was maintained at 1 ml/min and elute was monitored at 280nm for proteins and 340nm as specific signal for blue oxygen carrier protein *i.e.* hemocyanin. Likewise, samples were also subjected to anion exchange chromatography using Mono Q 5/50GL FPLC column (Amersham Biosciences, UK) equilibrated in 20mM imidazole-HCl buffer, pH6.5 containing 1mM $CaCl_2$ and 0.5mM $MgCl_2$ (buffer A). Elution was performed with in buffer A additionally containing 500mM NaCl (buffer B) using the following gradient program; 0% B for 5 min; 60% B in 10min; 100% B in 2min; and finally washing with 100% B for 2min. The flow rate was maintained at 1ml/min and the elute was monitored at 280 and 340nm. Comparative analysis of the separation profiles of three *Siphonaria* species was performed using the software UNICORN 5 (Amersham Biosciences, UK).

Statistical analysis

The difference in total protein, lipid and carbohydrate contents of *S. ashgar, S. belcheri* and *S. kurracheensis* were analyzed by one-way ANOVA.

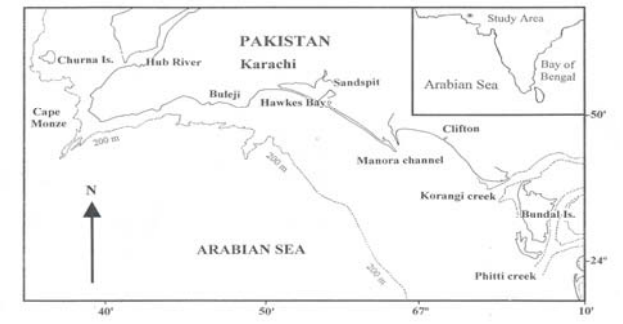
RESULTS AND DISCUSSION

Species of Siphonaria

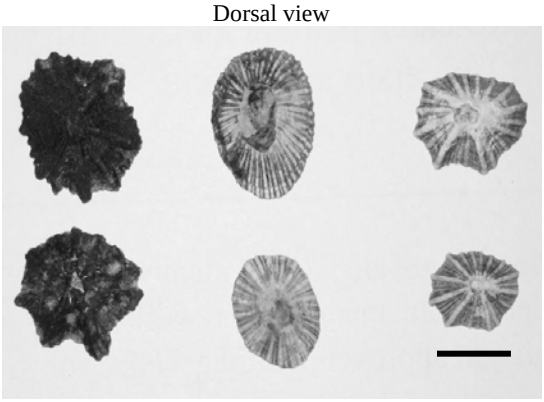
The species of *Siphonaria* were identified on the basis of shell color and structure, and were found to comprise three species, *Siphonaria asghar, S. belcheri* and *S. kurracheensis*. The description of the three species (Fig. 1B) is given in Bano *et al.* (2011). The specimens used for present study had average length in *S. ashgar*, 14.2±2.7 mm, in *S. belcheri*, 13.9±2.2 mm and in *S. kurracheensis* 14.7±1.9 mm.

Quantitative estimation of major macromolecules

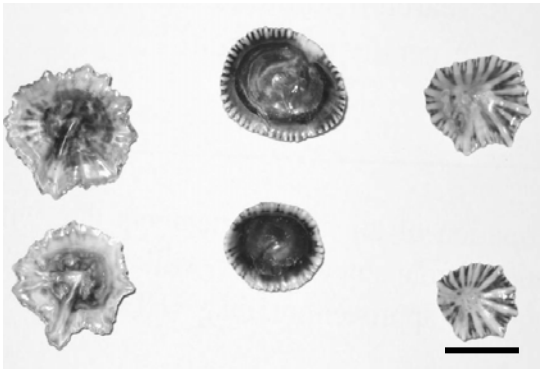
The protein, lipid and carbohydrate contents in the whole tissue of three species of *Siphonaria* are presented in Figure 2. The highest concentration of protein content was observed in *S. belcheri* (107.1 mg g^{-1}). No significant difference (F= 0.409; df= 2; P= 0.695) was found in the concentrations of tissue protein in the three species. The highest concentration of tissue lipid was observed in *S. kurracheensis* (92.5 mg g^{-1}). However, no significant difference (F=0.169; df= 2; P= 0.851) was found in the concentrations of tissue lipid in the three species. The carbohydrate content which was highest in *S. ashgar* (36.1 mg g^{-1}) also showed no significant difference (F=6.660; df= 2; P= 0.078) in carbohydrate concentrations in three species.



Dorsal view



Ventral view



S. kurracheensis ***S. ashgar*** ***S. belcheri***

Fig. 1. (A). Map showing the study area. (B). Dorsal and ventral view of the shells of *Siphonaria* sp., *S. kurracheensis, S. ashgar and S. belcheri.* Scale= 10mm (Photographed by SA Ali).

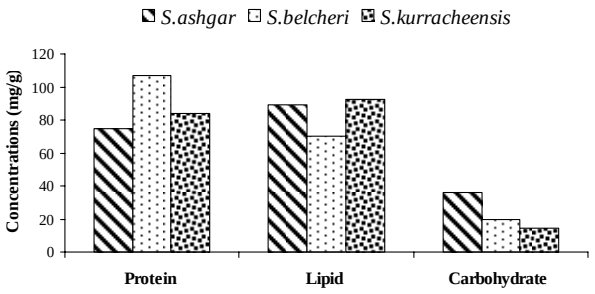


Fig. 2. Proximate composition of total protein, lipid and carbohydrates of three *Siphonaria* species.

Polyacrylamide gel electrophoresis

Approximately 20µg of crude protein extract of each tissue (tissue homogenates) was subjected to 8% SDS-PAGE (Laemmli, 1970) under dissociating (with SDS) and dissociating-denaturing conditions (with SDS and reducing agent DTT). Figure 3A shows the band pattern characteristic of *Siphonaria* hemocyanin, according to which under dissociating conditions one major band (*ca.* 400kDa) was observed which corresponds to two hemocyanin subunits (named as *Siphonaria* subunit S1 and S2). Under reducing conditions this band splits into three major bands, one major approximately 400kDa corresponding to the subunit S1, while S2 splits to produce two *i.e.* a 280kDa fragment S2.1 (corresponding to six-FUs) and the other 100-120kDa fragment S2.2 (corresponding to two-FUs) as reported for many other marine gastropods (Gebauer *et al.*, 1999). 6% Native gel electrophoresis on the other hand, was found to be more informative with reference to non-respiratory proteins (NRPs) in the three *Siphonaria* species (Fig. 3B). In this case as besides one major band of native hemocyanin (S1+S2; suggesting the presence of single isoform like hemocyanin from marine gastropod *Rapana thomasina*), partially dissociated hemocyanin bands and/or other non-respiratory proteins (NRPs) were also observed (Swerdlow *et al.*, 1996; Gebauer *et al.*, 199[illegible]lakieva *et al.*, 2000).

The structure and function of hemocyanin have been intensively studied in cephalopods and gastropods, and they are also found in chitons and some bivalves (Van Holde and Miller, 1995). The basic structure of all known hemocyanins is

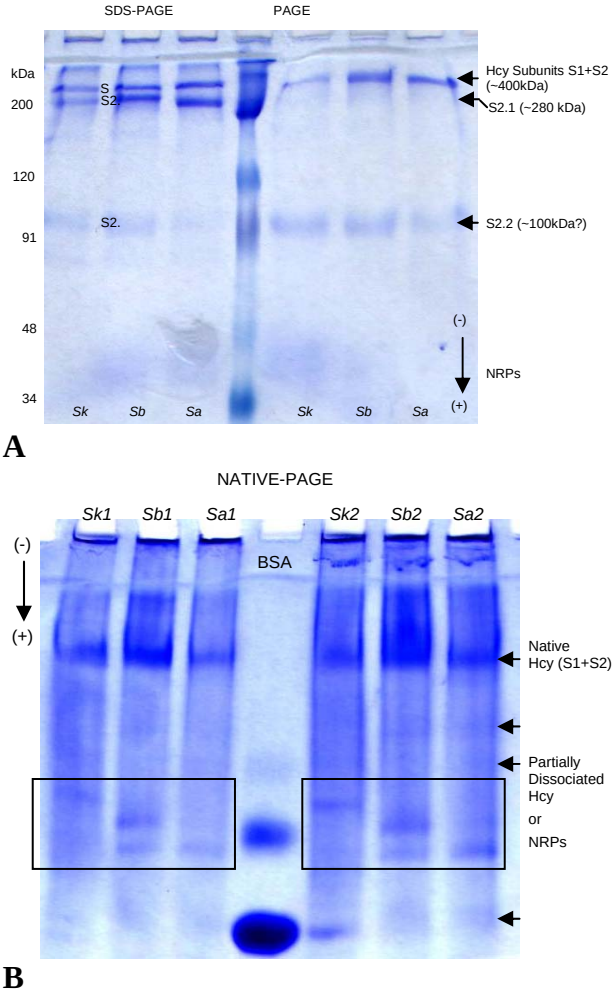


Fig. 3. Polyacrylamide gel electrophoresis patterns of homogenate preparations of the three identified *Siphonaria* species. (A) 8% SDS-PAGE (B) 6% native gel electrophoresis of the same.

decameric. Cephalopod and chiton (*Octopus dofleini*, *Sepia officinalis* and *Lepidochiton* sp.) hemocyanins are organized as decamers, consisting of ten identical subunits, with a molecular mass of 350 or 400 kDa and folded into seven or eight functional units with molecular mass of about 50 kDa (Herskovits and Hamilton, 1991; Van Holde *et al.*, 1992; Markl *et al.*, 2001). In gastropods and bivalves, hemocyanins are arranged as di-decamers (*i.e.* two decamers or having two immunologically distinct subunit types). This type of hemocyanin has been characterized in the vineyard pulmonate snail *Helix pomatia* and prosobranch gastropod *Rapana thomasiana* (Gebauer *et al.*, 1999). In addition,

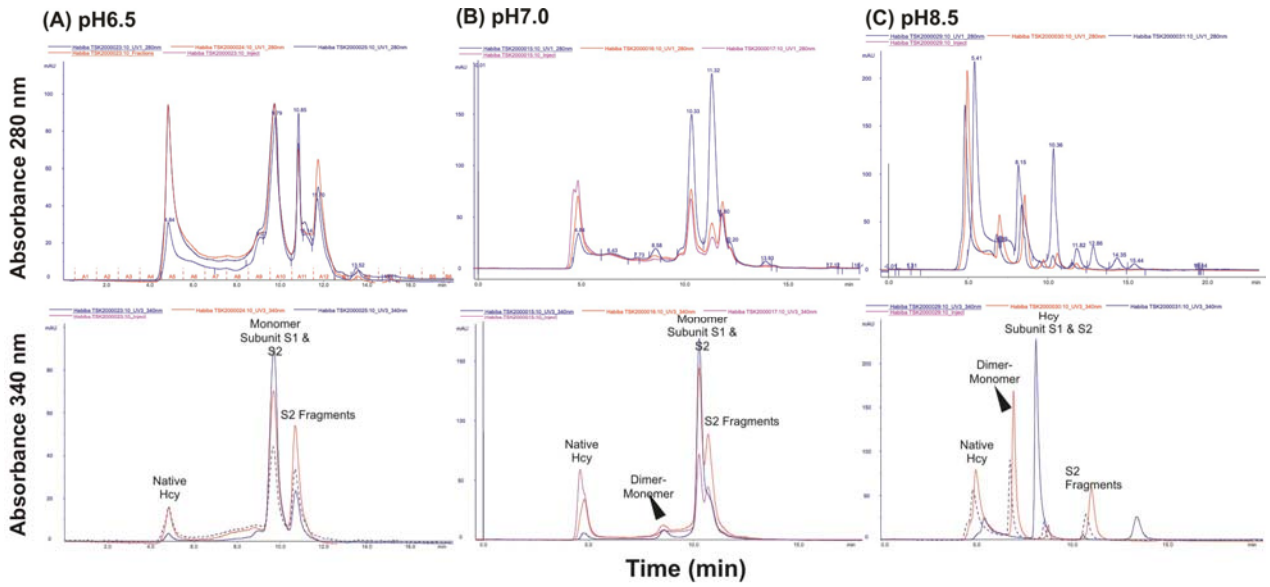


Fig. 4. Comparative chromatographic behavior of the homogenate preparations of the three identified *Siphonaria* sp. by SEC-FPLC. SEC column was equilibrated and eluted with (*A*) 20mM Imidazole-HCl buffer, pH6.5 containing 1mM $CaCl_2$ and 0.5mM $MgCl_2$, and (*B*) 100mM Phosphate buffer, pH7.0, (*C*) 50mM Tris-HCl buffer, pH8.5. The flow rate was maintained at 1 ml/min and the eluate was monitored at 280nm for proteins and 340nm as specific signal for blue oxygen carrier protein hemocyanin. *Note:* despite identical separation profiles at specific wavelength 340nm, dissociating pattern of hemocyanin is different at different pH values. Color tracing: Blue, *S. ashgar*; Red, *S. belcheri* and Black/Magenta, *S. kurracheensis*.

some molluscan hemocyanins further aggregates even larger than di- tri-decamers or multi-decamers (Lieb *et al*., 2010). Such oligomeric forms are the hemocyanin of bivalve, *Yoldia thraciaeformis* and keyhole limpet, *Megathura crenulata* (Gebauer *et al*., 1994; Swerdlow *et al*., 1996; Harris and Markl, 1999; Markl *et al*., 2001).

SEC/IEX - fast protein liquid chromatography

Change in physiological pH was found to be one of the most defining factors in the aggregation level (*i.e.* multi-decamers, di-decamers and decamers etc), dissociation and reassociation equilibrium (*i.e.* ratio between the different oligomers, subunit dimmer, and monomer) and consequently the oxygen binding properties in gastropod hemocyanins (Herskovits *et al*., 1990, 1992; Taiwo, 1992; van Holde *et al*., 1992). For example in keyhole limpet (KLH) two hemocyanin isoforms exist and it has been reported that at physiological pH7.0 the KLH2 multi-decamers had a tendency to dissociate into decamers and into subunits when the pH was further reduced to pH 5.7–5.9. On the other hand, KLH1 exists predominantly as individual di-decamer, which remains intact and thus can be separated by size exclusion chromatography (Harris and Markl, 1999). Similarly, anion ion-exchange chromatography may also be utilized for the purification of the two hemocyanin subunit types in oligomeric state but with relatively lesser reproducibility (Swerdlow *et al.*, 1996; Gebauer *et al*., 1999; Schütz *et al*., 2001). The existing knowledge not only enables us to achieve separation and characterization of the hemocyanin by SEC/IEX chromatography but also to trace the subtle differences in the unknown hemocyanin of *Siphonaria* species.

Protein sample preparations of three *Siphonaria* species, after dialysis at different pH values, were first subjected to size exclusion fast protein liquid chromatography. Figure 4A shows the separation profiles under buffering condition of 20mM imidazole-HCl buffer, pH 6.5 containing

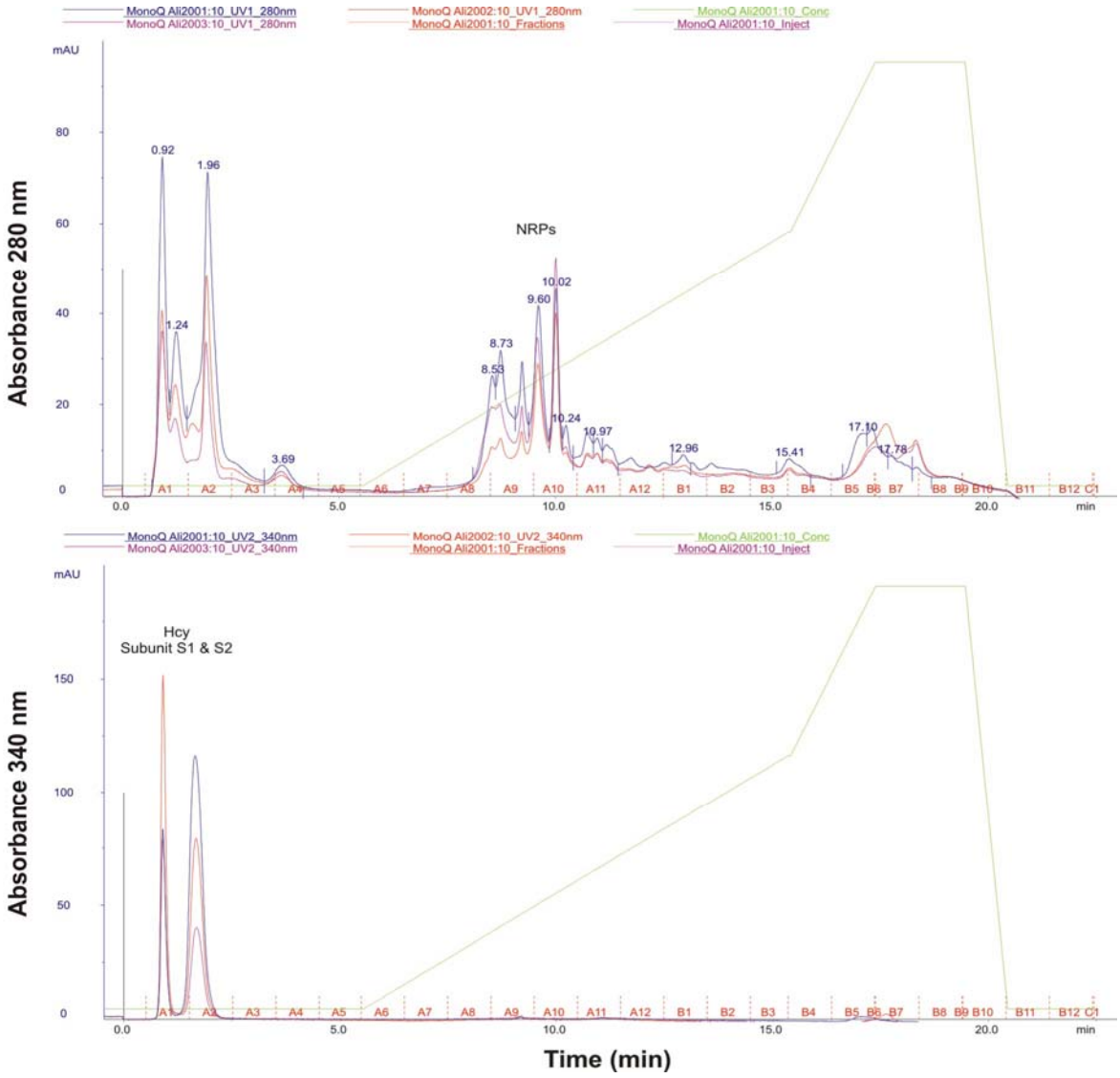


Fig. 5. Comparative chromatographic behavior of the homogenate preparations of the three identified *Siphonaria* sp. by IEX-FPLC. Anion exchange Mono Q column was equilibrated in 20mM Imidazole-HCl buffer, pH6.5 containing 1mM $CaCl_2$ and 0.5mM $MgCl_2$ (buffer A). Elution was performed using 500mM NaCl in A (buffer B) using the following gradient program; 0% B for 5 min; 60% B in 10min; 100% B in 2min; and finally wash in 100% B in 2min. The flow rate was maintained at 1ml/min and the eluate was monitored at 280 and 340nm. Color tracing: Blue, *S. ashgar*; Red, *S. belcheri* and Magenta, *S. kurracheensis*.

1mM $CaCl_2$ and 0.5mM $MgCl_2$, a condition commonly used for the purification of two hemocyanin subunits *e.g.* keyhole limpet hemocyanin (Swerdlow *et al.*, 1996; Schütz *et al.*, 2001). Due to the close molecular masses, the two subunits (S1 and S2) eluted in the same peak along with partly dissociated fragments from subunit S2 (S2.1-2) which are clearly separated from un-dissociated native material. Further increase in the pH to 7.0 and 8.5 enhances the dissociation and ultimately the different dissociation pattern and equilibrium between native oligomer, dimer-

monomer and monomer hemocyanin (Figs. 4B-C). Despite very similar and/or overlapping separation profiles of the hemocyanin preparation from three *Siphonaria* species, sharp differences can be seen in terms of dissociating pattern, oligomeric equilibration, and the ratio of the different components at specific wavelength *i.e.,* 340nm. In order to complement SEC results samples were also subjected to ion-exchange chromatography under ideal dissociating conditions, results revealed two specific major peaks in the unbound region corresponding to the two hemocyanin subunits (Figure 5). This is unlike the other reports on gastropod hemocyanins (Swerdlow *et al*., 1996; Gebauer *et al*., 1999; Schütz *et al*., 2001) and probably arises from the method of preparation of hemocyanin from tissue instead of isolated hemolymph of *Siphonaria* species.

Subtle variation in terms of peak and band intensity as well as fragmentation pattern of hemocyanin subunits in three phenotypically different *Siphonaria* species also suggest the expression of different ratio of subunits and consequently their role in the formation of different oligomers and/or higher aggregates. Harris *et al*. (1997) in a comprehensive analysis of 325 individual keyhole limpets have also observed an average ratio of 1:2 for the two subunit types (i.e. KLH1 and KLH2). Likewise, partial if not complete depletion of KLH1 (without effecting KLH2) was also reported during the prolong period of animal captivity suggesting the involvement of hemocyanin in ecophysiological adaptation (Markl *et al*., 1991; Harris *et al*., 1997; Söhngen *et al*., 1997). Recently Streit *et al.* (2005) in a developmental study on gastropods (*Haliotis asinine*) have also observed that the two hemocyanin genes which encodes hemocyanin isoforms HaH1 and HaH2 were tissue specific and differentially expressed in the animal supporting the idea that each isoform has a unique role beyond the oxygen transport function in the hemolymph. In conclude, the subunit composition of hemocyanin from *Siphonaria* resembles to the hemocyanin of other reported gastropods and thus could also be subjected for further immunological investigations for their possible biomedical application.

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