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Physical, biochemical and functional characterization of haemoglobin from three strains of *Artemia*

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Abstract

The brine shrimp, *Artemia,* an inhabitant of coastal and inland salterns, encounter fluctuations in the salinity which in turn influences the oxygen availability of their habitat. Hence, experiments were performed to analyze variations in haemoglobin structure and patterns of three strains of *Artemia* from South India and also to reflect the effect of varying oxygen levels in their habitat. Haemoglobins were purified on a DEAE–Sephadex column and haemoglobin types were analyzed by comparing their relative mobility on a non-denaturing medium. Furthermore, their molecular masses were determined by gel filtration in Sepharose column and by dodecylsulfate polyacrylamide gel electrophoresis. Results clearly reveal the presence of three distinct extracellular haemoglobins Hb I, Hb II and Hb III in Tuticorin strain while the other strains displayed only trails or the complete absence of Hb III and Hb II. Estimated molecular masses of these haemoglobins are 235,000–250,000 Da. Denaturation of the reduced and alkylated haemoglobins revealed apparently one polypeptide chain with a molecular mass of 124,000 Da. Upon denaturing gel electrophoresis of native haemoglobin Hb II, it was found that the 124,000 Da, polypeptide was cleaved specifically into two unequally-sized fragments of 50,400 and 79,800 Da. With regard to oxygen affinity, Hb III has a very high affinity for oxygen, an almost negligible Bohr effect and a good physiological adaptation to temperature changes. By combining the three haemoglobins in different proportions *Artemia* strains must be able to withstand diverging environmental conditions. In particular, the absence of Hb III in Puthalam and its occurrence as a faint band in Thamaraikulam could be correlated to the oxygen levels of their habitats.

Keywords: *Artemia*; Electrophoresis; Gel filtration; Haemoglobin; Oxygen affinity; South India

1. Introduction

Haemoglobins play a key role in the evolutionary balance between nutrient availability and the regulation of aerobic metabolism and in supplying increased amounts of oxygen at lower internal oxygen partial pressures. Thus, there is an evolutionary balance between the investment of additional nutrients into the synthesis of haemoglobin and the reduction of stress on electron transport systems and related metabolic pathways. Brine shrimps inhabit an ecological niche which shows a great diversity in environmental factors to which they must show a variety of adaptations. One of these is the development of high concentrations of haemoglobins in hypoxic conditions. The haemoglobins of brine shrimp may prove useful for studying regulatory mechanisms in higher animals. The haemoglobins are inducible (Bowen et al., 1966) and shrimp are easily cultured for use in genetic studies (Bowen et al., 1969).

The extracellular haemoglobins of invertebrates can be grouped into three categories according to their molecular size (a) those with a high molecular mass ($1.5–3.8\times10^6$) found in molluscs and annelids (Wood and Mosby, 1975), (b) those with a medium molecular mass ($2.2–4.5\times10^5$) mostly detected in crustaceans (Waring et al., 1970; Hourdez et al., 2000; Kato et al., 2001) and (c) some with very low molecular mass ($1.6–3.2\times10^4$) found in *Chironomus* species (Svedburg and Eriksson, 1933). *Artemia* haemoglobins belong to the second group by their molecular mass ($2.5–2.6\times10^5$). *Artemia* haemoglobins have been reported to contain three polypeptide chains with approximately molecular masses of 50,000–54,000,

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Table 1
Environmental parameters in the three locations chosen for the present study

Parameters	Tuticorin	Thamaraikulam	Puthalam
Temperature (°C)	29.5±5.55	25±2.11	24.00±2.1
Salinity (‰)	101.00±2.50	67.5±2.26	55.5±3.05
Dissolved Oxygen (mg/l)	3.30±0.10	4.50±0.24	5.90±0.30
pH	9.00±0.02	6.9±0.02	8.78±0.04

$\bar{X}$±S.D of 30 observations.

80,000–90,000 and 110,000–130,000 Da, *in vitro* by a specific cleavage and that *Artemia* haemoglobins are in the form of a dimer composed of two large globin subunits that are analogous to subunits of molluscan haemoglobins (Gallardo and Castro, 1987).

The entomostracan crustaceans contain two of the better-known sets of examples of adaptive haemoglobin synthesis: (a) Several species of *Daphnia* and some related freshwater cladocerans change from colourless to bright red with haemoglobin production in response to hypoxia (Fox, 1948; Hoshi et al., 1969; Smaridge, 1956). (b). The brine shrimp, *Artemia*, an anostracan responds to either decreased oxygen partial pressure or to increased salinity by haemoglobin synthesis (Gilchrist, 1954; Bowen et al., 1966; Wolf et al., 1987; Helland et al., 2000). *Artemia* populations in salt lakes or coastal salterns may vary greatly in phenotype (type and amount). In one saltern, on San Francisco Bay, adults were found to have Hb II and Hb III (in both sexes). In an adjacent saltern, all females had both Hb I and Hb II whereas, males had only Hb II (Bowen et al., 1966). Evidence for environmental determination of phenotype has been seen with an increase of total haemoglobin concentration of haemolymph in response to low oxygen, high CO, ferric EDTA and certain amino acids or vitamin. Further evidence is the presence of three different haemoglobin phenotypes in genetically identical adult females from one parthenogenetic clone. During larval development, *Artemia* show progressive change in the type of haemoglobin, which suggests that ontogenetic factors also influence phenotype. Observation on three parthenogenetic and eight zygogenetic populations suggested that the three haemoglobins were present in most of the *Artemia* populations. Fourth haemoglobin (Hb X), which may be a variant of one of the three more common haemoglobins has been reported (Bowen et al., 1966). The three most negatively charged haemoglobins in the haemolymph are designated Hb I, Hb II and Hb X in descending order of mobility in electrophoresis at pH 8–10 on non-sieving and sieving support media (Gratzer and Beaven, 1961; Bowen et al., 1966). These proteins are, respectively, the Hb I, Hb II and Hb III of Moens and Kondo (1976) and Heip et al. (1980).

The haemoglobin concentration fluctuates mainly according to the dissolved oxygen concentration in the water in which these animals live and an inverse correlation has been reported to exist between the dissolved oxygen concentration and haemoglobin concentration (Kobayashi, 1981, 1982; Guadagnoli et al., 2005; Hourdez and Weber, 2005). This paper examines variations in the haemoglobin pattern among a bisexual strain inhabiting a coastal saltern in Tuticorin (08°48' N 78°11' E) and two parthenogenetic strains inhabiting the inland salterns of Puthalam (09°83' N 79°30' E), and Thamaraikulam (09°39' N 78°30' E) of South India, as these strains inhabit areas with significant variations in environmental parameters such as salinity, oxygen, temperature etc. (Table 1). This study is an effort towards the functional characterization of these three haemoglobins together with influence of some environmental factors on oxygen affinity.

2. Materials and methods

2.1. Preparation of haemoglobin

Separation of haemoglobin was carried out as per the methodology of Azem and Daniel (1992). About 100 adult brine shrimps of each strain harvested from their respective salterns were rinsed in double distilled water, suspended in 0.1 M Tris–HCl buffer pH 7.7 containing 2.6 mM phenylmethanesulfonyl fluoride, 20 ng/mL soybean trypsin inhibitor and 1 mM EDTA, referred to hereafter as extraction buffer. The suspension was homogenized and the homogenate was centrifuged for 20 min. at 1600 *g* to remove particulate matter. Centrifugation was repeated for 4–6 times until a clear fluid was obtained. Clear fluid was centrifuged at 105,000 *g* for 6 h in a Beckman LE-80 Ultracentrifuge, USA. The supernatant was discarded and the precipitate was dissolved in the same extraction buffer. This extract was again centrifuged at 105,000 *g* for 6 h and the supernatant was discarded. The resulting red precipitate was collected and redissolved in extraction buffer.

2.2. Purification of haemoglobin

Crude haemoglobin was further purified by DEAE–Sephadex chromatography as per the methodology of Moens and Kondo (1978). The pellet resuspended in extraction buffer was fractionated on a column of DEAE–Sephadex A-50 (20×2.5 cm) equilibrated with 50 mM Tris–HCl (pH 7.5). Haemoglobin eluted with buffer containing 225 mM NaCl was detected at 410 nm.

2.3. Non-denaturing gel electrophoresis

The pooled and concentrated fractions were electrophoresed on a non-denaturing medium to localize the haemoglobin subtypes. Samples containing 30 μg haemoglobin were applied to the wells on a slab of polyacrylamide gel gradient (2.6–28%) and electrophoresed in non-denaturing medium for 9 h at 90 V with bovine serum albumin as reference protein as per the methodology of Laemmli (1970). Samples were electrophoresed until the bromophenol blue marker was about 0.5 cm from the edge of the gel. The gels were stained with Coomassie brilliant blue G-250 for 1 h to locate the haemoglobin bands in one of the tracks for the three respective strains. The haemoglobin bands in the remaining tracks were excised, finely sliced and eluted into a minimal volume of extraction buffer. The eluate was centrifuged at 90,000 *g* for 6 h and the red pellet was dissolved in the extraction buffer.