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Isolation of neurosecretory hyperglycemic hormone from the eyestalks of freshwater crab, *Barytelphusa cunicularis*

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Abstract. A procedure is described for the purification of neurosecretory hyperglycemic hormone from the eyestalks of freshwater crab, *Barytelphusa cunicularis*. This procedure involved a combination of gel filtration on Sephadex G-50 and a subsequent preparative polyacrylamide gel electrophoresis which revealed that hyperglycemic hormone moved faster than other proteins.

Keywords. Hyperglycemic hormone; eyestalk; purification; *Barytelphusa cunicularis*.

1. Introduction

Neurosecretory sinus gland complex in the crustacean eyestalk produces, stores and releases a number of hormones which regulate diverse physiological processes. Substantial progress has been made in the complete elucidation of the structures of two eyestalk hormones—red pigment concentrating hormone (Fernlund and Josefsson 1972; Fernlund 1974) and distal retinal pigment light adapting hormone (Fernlund 1971, 1976) in the prawn, *Pandalus borealis*. Hyperglycemic hormone (HGH) is the next most intensely studied eyestalk hormone (Kleinholz and Keller 1973; Kleinholz 1975, 1976). Abramowitz *et al* (1944) first described the diabetogenic action of the eyestalk extract. They found that sinus gland extract of *Uca pugilator* and *Callinectes sapidus* had the effect of increasing blood sugar level in these crabs. Kleinholz and Little (1949) found that such increases in total reducing substances were true hyperglycemias, due to fermentable sugar. HGH has been characterized as a protein of relatively small molecular weight, with an average of 6600 dalton, and several purification steps have been described (Kleinholz *et al* 1967; Keller 1968; Kleinholz and Keller 1973; Skorkowski *et al* 1977). Comparative electrophoretic studies of HGH from the crab, *Cancer magister*; the prawn, *Pandalus borealis* and the crayfish, *Orconectes limosusall* indicate the possibility of species difference in molecular structure of crustacean HGH (Kleinholz and Keller 1973). HGH from 3 different species have different electrophoretical mobilities (Kleinholz and Keller 1973; Keller 1977). Kleinholz (1975) and Keller and Wunderer (1978) have reported the amino acid composition of HGH from *C. magister* and *Carcinus maenas* respectively. Luven *et al* (1982) discussed species or group specificity in biological and immunological studies of crustacean hyperglycemic hormone. Martin *et al* (1984) have isolated and characterized HGH in a terrestrial isopod, *Porcellio dilatatus*. Since most of the work mentioned above is on marine crustaceans, an attempt has been made to isolate HGH from the eyestalks of the freshwater crab, *Barytelphusa cunicularis*.

2. Materials and methods

B. cunicularis were collected from Kham river near Aurangabad and acclimatized to

the laboratory condition. They were fed with pieces of earthworms twice a week and food was withdrawn 24 h before the commencement of the experiment.

2.1 *Extraction*

Sixty eyestalks were removed from mature intermoult crabs, irrespective of their sex. Cuticle and most of the non-nervous tissues were carefully removed. The eyestalks were homogenized thoroughly in 2 ml ammonium acetate buffer (pH 8·5, 50 mM); homogenate was centrifuged for 30 min at 20,000 *g* in a K-24 refrigerated centrifuge at 4°C. The supernatant was collected and the residue was re-extracted with 1 ml of the acetate buffer and again centrifuged for 30 min. The supernatants were pooled together. This supernatant (2 ml) contained 17 mg proteins (Spector 1978).

2.2 *Gel filtration*

A Sephadex G-50 (superfine) column (1·2 × 64 cm) was used for the separation of HGH, the column was equilibrated with ammonium acetate buffer (pH 8·5, 20 mM). The clear supernatant was applied to the column and elution was carried out with a flow rate of 10 ml/h. Fractions of 1 ml were collected and the protein content and hyperglycemic activity of each fraction were determined.

2.3 *Electrophoresis*

Polyacrylamide gel electrophoresis (PAGE) was carried out in Bronga slab electrophoresis apparatus (Balaji Scientific Service, Madras) using a gel of 7·5% acrylamide at pH 8·9 (Tris-glycine buffer) as described by Davis (1964). The thickness of gel was 15 mm and measured 18 × 18 cm. Samples obtained from the column showing maximum hyperglycemic activity (fraction nos. 43–45) were dialysed against Tris-glycine buffer and were carefully applied with the help of microsyringe into different wells of the gel. Bromophenol blue was used as a tracking dye. Electrophoresis was carried out by applying a current of 12 mA at 8°C until the tracking dye migrated 2–3 cm from the lower end of the gel. A portion of gel containing two wells used for ascertaining the electrophoretic mobility of the applied sample and the remaining major portion used to extract the hyperglycemic activity. The gel was stained with Coomassie brilliant blue R 0·25% in methanol/water/acetic acid (227:227:46, v/v) and destained with sereal changes of methanol:acetic acid:water (10:3:35, v/v). The rest of the gel was cut transversely into 4 segments (3 segments containing 3 band and fourth without any band) based on localization of the bands in the stained gel. Each segment of the gel was separately homogenized in 2 ml of chilled distilled water centrifuged at 4°C for 10 min. The HGH activity was tested in the supernatant.

2.4 *Bioassay*

Crabs of either sex of size 5 × 3 cm were selected. Eyestalks were removed bilaterally and the cut surfaces were cauterized to minimize bleeding. Such destalked animals,

starved for 3 days before use, served as both experimental and control animals. Concentrations of glucose in blood were determined by phenol sulphuric acid method (Dubois *et al* 1956). Blood samples were taken from each of the 6–8 destalked animals of experimental group immediately before and 90 min after the injection of 0·1 ml of test extract. Each animal thus served as its own control. Same procedure was followed with control animals where 0·1 ml of acetate buffer (column chromatography) or glass distilled water (electrophoresis) was injected.

2.5 *Protein determination*

Protein was determined by Coomassie brilliant blue dye procedure (Spector 1978) with bovine serum albumin as a standard protein.

3. Results

The elution of eyestalk extract on Sephadex G-50 column was resolved into 4 different protein peaks (figure 1). Hyperglycemic activity was observed in the II peak (fraction nos 41–47) while peak I (fraction nos 26–40), peak III (fraction nos 48–55) and peak IV (fraction nos 59–68) did not show any HGH activity. It suggests that the first peak corresponds to high molecular weight protein while peaks III and IV contain low molecular weight proteins as compared to HGH.

The eluted sample from Sephadex G-50 with HGH activity subjected to PAGE (pH 8·9) resolved into 3 bands (figure 2). The separate extraction of these bands for HGH assay revealed that HGH activity was associated with the intensely stained and well separated band No. 3 that moved faster than the other two bands (table 1).

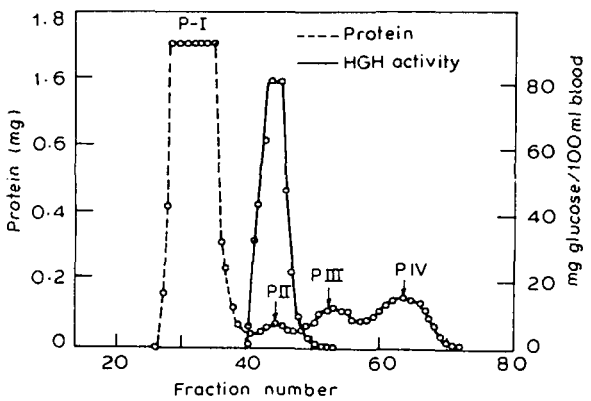

Figure 1. Chromatography of eyestalk extract in ammonium acetate buffer (pH 8·5, 50 mM) from the crab, *Barytelphusa* on Sephadex G-50 (superfine). The column was equilibrated with ammonium acetate buffer (pH 8·5, 20 mM); flow rate 10 ml/h; fraction value 1 ml; total volume 57 ml; void volume 21 ml. The eyestalk extract consisting of 17 mg in 2 ml was applied onto the column. The chromatographic activity of each fraction was tested on 10 animals/fraction and response is depicted in solid line, whereas, protein content is represented in dashed line. P–I, P–II, P–III and P–IV represent various protein peaks.

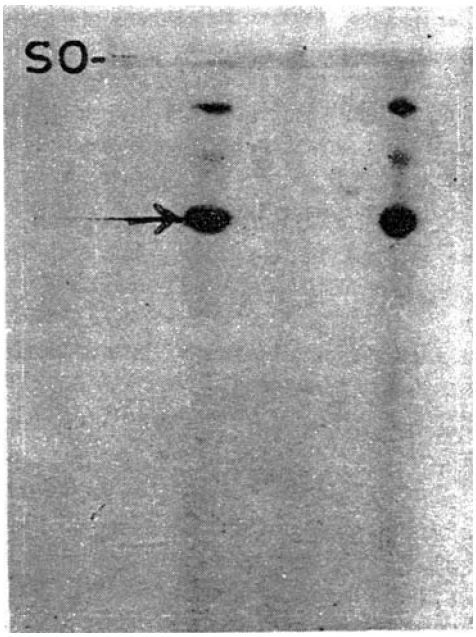

Figure 2. PAGE of the active HGH fraction from Sephadex G-50 conducted according to the method of Davis (1964). Arrow indicates band showing HGH activity. SO, sample origin.

Table 1. Effect of the supernatents of the PAGE segments on the blood sugar level in the destalked crabs, *B. cunicularis* (Dubois *et al* 1956).

Electrophoretic segment No.	Blood sugar level (mg% ± SD)	
	Before injection	After injection
1	7·0 ± 2·9	7·8 ± 8·7^{NS}
2	6·4 ± 3·0	6·9 ± 3·7^{NS}
3	7·5 ± 2·6	22·6 ± 8·2*
4	7·2 ± 2·3	7·8 ± 3·9^{NS}
Glass distilled water control	7·6 ± 1·8	8·6 ± 1·3^{NS}

NS, Not significant. *$P < 0{\cdot}005$.

4. Discussion

The present investigation was undertaken to develop a simplified procedure for the purification of HGH from the eyestalks of the crab, *B. cunicularis* and to confirm its identity with the other crustacean hyperglycemic hormones reported. Purification procedures of HGH from the eyestalks of marine prawn, *P. borealis* (Kleinholz *et al* 1967), shrimp *Crangon crangon* (Skorkowski *et al* 1978) and from the sinus glands of *C. maenas* (Keller and Wunderer 1977) have been reported, whereas, there is no information on the isolation and characterization of HGH in freshwater crustaceans. Initial characterization of HGH from eyestalk extract of *B. cunicularis* has been carried out.

Isolation of HGH from *B. cunicularis* by fractional precipitation with chilled acetone (−10°C) and different concentrations of ammonium sulphate have been tried but did not show any decided advantage because of the poor recovery of the final product. Our approach of the two step isolation procedure by gel filtration and PAGE has been helpful in the elimination of most of the other unassociated proteins as seen in figure 1. Further isolation of the preparation is achieved by electrophoresis and almost homogenous preparation in separate well defined band was obtained even though we have not further tried to purify this. The sinus gland is a much more suitable starting material for isolation of HGH than the whole eyestalk extract. Keller (1977) has shown that HGH is present rather in high quantities in the sinus gland (more than 10%) can be separated from other proteins in PAGE at pH 8·9.

Our efforts in the isolation of sinus glands from the eyestalks were unsuccessful and hence the starting material was contaminated with a number of other eyestalk tissue proteins. We have followed the procedure of Keller and Wunderer (1978) for the isolation of HGH from *B. cunicularis* but the order of technique used by them was reversed. Gel filtration employed first was helpful to characterize a large number of tissue proteins in the eyestalk extract (peaks I, III and IV) which failed to induce hyperglycemia in experimental crabs. HGH activity was associated only with the II protein peak. When PAGE of this II protein peak was carried out, HGH moved faster than other proteins. HGH was found to be associated with the strongest staining band. The presence of HGH activity of *B. cunicularis* in only one peak corroborates with similar activity associated with the HGH isolated from the eyestalks of *P. borealis* (Kleinholz *et al* 1967). Skorkowski *et al* (1977) observed HGH activity in two protein peaks by gel filtration of eyestalk on a Sephadex G-75 column and suggested that two molecular forms of HGH are present in *C. crangon*. The anionic behaviour of *B. cunicularis* and its occurrence in well separated and strongest stained band is in accordance with the reports of Keller (1977) and Skorkowski *et al* (1977).

We have not checked the homogeneity of the final preparation. Hence, further purification, determination of molecular weight, specificity and other properties need further investigation.

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