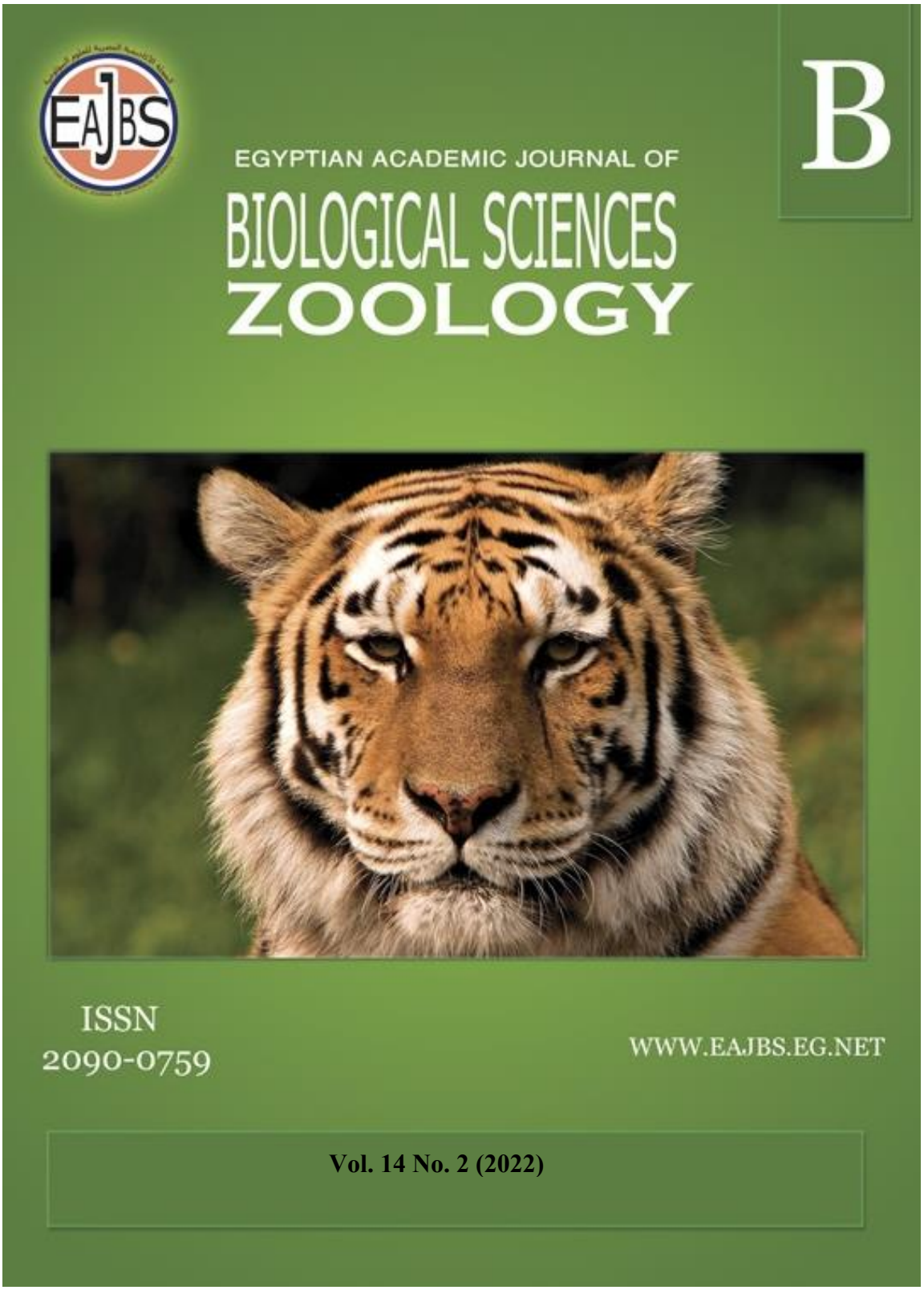

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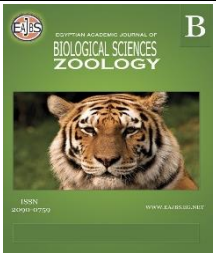

Survey of Natural Agglutinins in Two Species of Marine Crabs

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ABSTRACT

Agglutinins/lectins are conventionally defined as proteins/ glycoproteins of non-immune origin with a remarkable ability to specifically and reversibly interact with carbohydrate ligands. Lectins from different sources may essentially exhibit common biological activities. This study was therefore undertaken to survey naturally occurring agglutinins in two species of marine crabs by hemagglutination assay using mammalian erythrocytes. Hemagglutination assay results showed that the hemolymph of the marine crab *Grapsus albolineatus* showed the highest HA titer with rat erythrocytes. HA titer of the crab *Leptodius sanguineus* varied from 0 to 32 with all the tested erythrocytes. Among the various tissues of *Grapsus albolineatus* analyzed for the presence of agglutinins, hemagglutination activity was observed in the hemolymph and hepatopancreas with rat erythrocytes. HA was determined by both male and female crabs of *Grapsus albolineatus*. HA activity increased with an increase in animal size. Biochemical factors like water, protein and calcium content of the hemolymph did not have any influence on the HA titer.

INTRODUCTION

Invertebrate lectin seems to participate in the innate immune response by inducing bacterial agglutination or activation of phagocytosis through binding to sialic acids on foreign cells (Iwanga and Lee 2005). Invertebrate innate immunity relies on both cellular and humoral components. Humoral molecules are mainly found in hemolymph plasma and cell hemocytes. The hepatopancreas and hemocytes of crustaceans are regarded as the most important tissues involved in crustacean immunity (Grass *et al*., 2001). Lectins are proteins with diverse molecular structures that share the ability to recognize and bind specifically and reversibly to carbohydrate structures without changing the carbohydrate moiety. Lectin is described as a substance that can agglutinate cells or precipitate glycoconjugates, with a structure resembling a carbohydrate-binding protein or glycoprotein and is not of immune origin (Vasconcelos *et al.*, 2004). Due to its capacity to react selectively on a specific sugar group (Marques and Barraco 2000), lectins are considered a biomolecule of interest in glycobiology. Lectins have gained much attention for biomedical applications owing to their antimicrobial and anticancer potential (Silvester Mary Mettilda Bai *et al.*, 2022). Along with their biological role, they are documented as

one of the best biomolecules of medical interest, hence an attempt was carried out to survey agglutinin in two species of marine crabs.

MATERIALS AND METHODS

Experimental Crabs:

Two marine crabs, *Grapsus albolineatus* and *Leptodius sanguineus* were collected from Kadiyapatanam (8.1262°N latitude and 77.3196°E longitude) and Muttom (37.6428°N latitude and 78.3924°E longitude) coasts, Kanyakumari, Tamil Nadu, India, for this study.

Collection of Hemolymph and Separation Of Serum:

Hemolymph was collected by the method of Mercy and Ravindranath (1993).

Separation of Hemocytes:

The hemocytes from the hemolymph were separated using the method of Soderhall and Smith (1983).

Preparation of Tissues/Body Parts Extract:

Eyestalk, carapace, gills, muscles and hepatopancreas from the adult, healthy and non-autotomised intermoult crabs were dissected and rinsed in cold TBS (pH 7.5) to remove the adherent hemolymph. The extract of the body parts was prepared by homogenizing 100 mg each of eyestalk, carapace, gills, muscles and hepatopancreas in 1 ml of cold TBS and centrifuged at 4000 rpm for 10 minutes at 4°C and the supernatant was tested for HA activity.

Erythrocyte Collection:

Blood from mammals was collected in Alseivier's medium for hemagglutination assay. Human blood A, B and O (blood bank), Goat and pig blood (slaughter houses) and blood of other animals were collected from the veterinary hospital by venipuncture of the neck (cow, ox and buffalo), ear (rabbit), forearm (dog) and heart puncture (rat, and mice). Erythrocytes were collected directly in modified Alseivier's medium.

Hemagglutination Assay:

Hemagglutination assay was carried out as described by Ravindranath and Paulson (1987) to find out the presence of hemagglutinin and to know the erythrocyte specificity.

Effect of Size and Sex on HA Assays:

In order to understand the influence of size and sex on hemagglutinability, hemolymph samples collected from male and female crabs of varied sizes were analyzed for HA activity.

Biochemical Analysis:

Water Content:

A known quantity of hemolymph was dried in a desiccator. The difference between the wet weight and dry weight gave the amount of water present in the tissue (Passoneau and Williams 1953).

Calcium Content:

Hemolymph calcium was measured following the O-Cresolpthalein complex one method (Cohen and Sideman 1979).

Estimation of Protein:

The protein concentration was estimated by the Folin-Ciocalteu method (Lowry *et al*., 1951).

RESULTS

Natural Hemolymph Hemagglutinin in The Experimental Crabs:

The hemolymph of the two species of marine crabs *Grapsus albolineatus* and *Leptodius sanguineus* were tested using various mammalian erythrocytes. Hemagglutination was observed with a differentiation between the hemagglutinating activity of the two species and the kind of erythrocytes agglutinated. The hemolymph of the crab *Grapsus albolineatus* agglutinated erythrocytes in the order: rat > mice = goat > rabbit = buffalo = pig = human B = human O. No agglutinability was observed with Human A, cow, dog and ox erythrocytes. The HA titer of the crab *Leptodius sanguineus* varied from 0 to 32 with all the tested erythrocytes. Rabbit, buffalo and human O erythrocytes showed maximum agglutinability (32) with *L. sanguineus* hemolymph (Table 1). Hence, *Grapsus albolineatus* was selected for further study.

Table 1: Hemolymph hemagglutinin titer value of the tested marine crabs**.**

Erythrocytes (n=10)	HA titer	
	Grapsus albolineatus	***Leptodius sanguineus***
Rat	**2048±0**	4±0.9
Goat	16±3.2	16±3.2
Mice	16±0	2±0
Rabbit	2±0.8	32±0
Buffalo	2±0	32±0
Pig	2±0	8±0
Human B	2±0	16±5.1
Human O	2±0.8	32±0
Human A	0	8±0
Cow	0	0
Dog	0	2±0
Ox	0	0

n = number of animals tested.

Distribution of Hemagglutinin in Diverse Body Parts of the Marine Crab, *Grapsus albolineatus:*

The analysis of hemagglutinins in hepatopancreas, hemocyte, carapace, gills, eye stalk and muscles of the crab *Grapsus albolineatus* showed maximum activity in the hepatopancreas with rat erythrocytes. The hemagglutination titer value was low in the muscle, carapace and eyestalk (Table 2).

Table 2: Naturally occurring hemagglutinin in the tissues of the marine crab *Grapsus albolineatus.*

Erythrocytes (n=10)	Hepatopancreas	Hemocytes	Carapace	Gills	Eyestalk	Muscles
Rat	**256**±0	32±0	16±0	32±0	16±0	16±0
Rabbit	2±0	0	0	2±0	0	0
Goat	0	0	0	4±0.9	0	4±0
Mice	16±0	2±0	0	0	0	8±0
Buffalo	0	0	0	0	0	2±0
Pig	0	0	0	0	0	2±0.8
Human B	0	0	0	0	0	0
Human O	0	0	0	0	0	0
Human A	0	0	0	0	0	0
Cow	0	0	0	0	0	0
Ox	0	0	0	0	0	2±0

Influence of Size and Sex on HA Titer:

HA titer increased with an increase in body weight of both male and female crabs, *Grapsus albolineatus*. However, the hemagglutinability of the hemolymph agglutinin was uninfluenced by the sex of the animals (Table 3).

Table 3: Impact of size and sex on the HA titer of the hemolymph of the marine crab *Grapsus albolineatus*

Size of the crab Carapace length (cm) (n=10)	HA titer	
	Male	Female
3.0-4.0	1024	1024
4.0-5.0	1024-2048	1024-2048
5.0-6.0	2048	2048
6.0-7.0	2048	2048

Biochemical Constituents and HA Activity:

The quantity of biochemical parameters such as water, protein, and calcium of the hemolymph of the crab *Grapsus albolineatus* is given in Table 4.

Table 4: Quantity of water, protein and calcium in the hemolymph of the marine crab *Grapsus albolineatus*

Characteristic analyzed (n=5)	Quantity in Hemolymph
Water (%)	82±0.02
Protein (mg/ml))	23±0.71
Calcium (mM)	13±0.28
HA	2048

DISCUSSION

Lectins are carbohydrate-binding proteins that are highly specific for sugar groups of other molecules and so cause agglutination of particular cells or precipitation of glycoconjugates and polysaccharides (Rutishauser and Sachs 1975). A survey of agglutinins by hemagglutination assay revealed the presence of agglutinin in the hemolymph of crab *Grapsus albolineatus* and *Leptodius sanguineus*. The maximum hemagglutinating activity was found in *G. albolineatus* with rat erythrocytes. The specific HA affinity of the hemolymph of crab *G. albolineatus* to rat erythrocytes suggests that the receptor determinants of rat erythrocytes are specifically recognized by the hemolymph agglutinin (Hakamori 1973). The lectins in the hemolymph of marine crustaceans are diverse as the binding specificity to cell surface glycoconjugates of mammalian erythrocytes, vary and are expressed as hemagglutination activity. Mouse and rat erythrocytes are confirmed to a general N-Acetyl glucosamine configuration as well as glucosamine configuration (Cornick and Stewart 1973), and also these erythrocytes contain NeuGc/NeuAc/4(7)-O-acetylated sialic acids (Bhavanandan *et al.*, 1964). Similar findings were reported in other crustaceans, *Varuna litterata* (Prakash-Shoba and Basil Rose 2016), *Atergatis integerrimus* (Elayabharathi *et al.*, 2020), *Lamella lamellifrons* (Bai and Rose 2020) which suggests that the erythrocytes types agglutinated by the hemolymph of the crab *Grapsus albolineatus* probably share a common surface receptor, but with a quantitative difference in HA binding sites.

The serum of the marine crab *L. sanguineus* was found to contain naturally

occurring agglutinin which reacts with blood type with diverse specificity. The serum showed the highest reactivity with rabbit, buffalo and human O erythrocytes. The receptor found on the glycocalyx of rabbit erythrocytes was reported to be N-acetyl neuraminic acid and 9-O-acetyl neuraminic acid (Hakamori 1973; Pfeil 1980). Human A, B, O, cow and goat erythrocytes contain NeuAc or NeuGc with α 2-3 linkage (Yamamoto *et al.*, 1981). The dog erythrocyte membrane contains a mixture of N-acetyl and N-glycolyl neuraminic acids (Yasue *et al.*, 1978). The importance of agglutinin to agglutinate some mammalian erythrocytes suggests the lack of specific cell receptors on these erythrocyte membranes. The HA assay revealed that the *G. albolineatus* hemagglutinin may be specific to N-acetyl glucosamine as evidenced by its affinity to rat erythrocytes.

Among the various tissues of *G. albolineatus* screened for HA activity, hepatopancreas showed remarkably high HA titer with rat erythrocytes. The high specificity of both hemolymph and hepatopancreas to rat erythrocytes evidenced that the hemagglutinin observed in the hemolymph and hepatopancreas may be the same. The Hepatopancreas, equivalent to the fat body of insects and the liver of mammals, was considered the most important tissue synthesizing proteins involved in the immune system of crustaceans (Gross *et al.*, 2001). The occurrence of agglutinins in the hepatopancreas has also been reported in other invertebrates such as *Fenneropenaeus chinensis* (Sun *et al.*, 2008; Wang *et al.*, 2009) *Penaeus monodon* (Ma *et al.*, 2008).

The hemagglutinating activity of both male and female crabs of the same size was similar. HA titer was unaffected by the sex of the animal *G. albolineatus*. It showed that sex had no influence on hemagglutinin. The hemagglutinability of *G. albolineatus* increased with an increase in the size of the species. This increase in HA titer with an increase in the size of the body revealed that these grown-up crabs are exposed to many pathogens, which in turn urge the animal to synthesize more hemagglutinin. Though there was variation in the content (protein, Ca^{2+} and water), HA titer was the same in all the tested animals. This confirmed that the biochemical parameters of the serum had no influence on the HA titer. The biochemical constituents of the animal are known to vary with season, size of the animal, stage of maturity and availability of food (Akbar *et al.*, 1988; Soundarapandian and Ananthan 2008).

CONCLUSION

In invertebrates, lectins have been reported to contribute to innate immune responses, including prophenoloxidase activation, enhancement of encapsulation, module formation of hemocytes, opsonisation, antibacterial activity, antifungal activity and injury healing. Lectins that specifically recognize various sialic acids and their carbohydrate-binding patterns can be used as a tool for identifying various sialyl epitopes in the field of cancer research and therapy. This study concluded the presence of natural agglutinin specific to rat erythrocytes in the hemolymph of the marine crab *G. albolineatus*, which can be further tapped for biomedical applications.

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