1.0 Preliminary characterization of agglutinins naturally occurring in the serum of Scylla serrata. 88

2.0 Isolation and characterisation of multiple agglutinins from the serum of Scylla serrata. 92

3.0 Functional analyses of isolated multiple serum agglutinins in cellular immune responses of Scylla serrata. 100
IV. DISCUSSION

1.0 PRELIMINARY CHARACTERISATION OF AGGLUTININS NATURALLY OCCURRING IN THE SERUM OF SCYLLA SERRATA

Naturally occurring agglutinins in the serum of the marine crab *S. serrata* have been studied previously by different investigators, which mainly involved detection and isolation of the humoral agglutinins. In these independent studies, the isolated agglutinins have been reported to express specificity for Neu5Gc (Mercy & Ravindranath, 1992) and sialoglycoconjugates such as fetuin (Chattopadhyay & Chatterjee, 1993) or BSM (Kongtawelert, 1998). Although the actual carbohydrate structures recognized by the two agglutinins reactive with sialoglycoproteins remain unresolved, these findings from different laboratories, suggest the possible occurrence of at least three agglutinins in the serum of *S. serrata*. The role of Neu5Gc-specific agglutinin in hemocoelic clearance of foreign materials has been investigated by Mercy & Ravindranath (1994). But the participation of two other agglutinins in immuno-defense functions of *S. serrata* has not been examined. In accordance with the objective of this study, we were interested to isolate these agglutinins in active form in order to examine *in vitro* their involvement in hemocyte-mediated cellular immune reactions. As a prerequisite for isolation and purification of the humoral agglutinins, we have made a preliminary study involving characterisation of the agglutinating activity in the serum of *S. serrata*. 
The serum of this crab was found to agglutinate various mammalian erythrocytes with high reactivity against rabbit, mouse, human B, and rat RBC and these results are in agreement with those from the previous study (Mercy & Ravindranath, 1992). The serum agglutinating activity was high within a pH range of 7.0 to 8.5, and lacks specificity for human blood group, a feature that appears to be common among crustaceans (Smith & Chisholm, 1992). Since the cross-adsorption tests performed by Mercy & Ravindranath (1992) demonstrated the efficiency of human B RBC in adsorbing from serum all agglutinating activity for this and other RBC types, human B RBC was used as suitable indicator cell type for all subsequent experiments performed in this study.

Several studies on the humoral agglutinins in crustaceans revealed that their activity is dependent on divalent cations, usually calcium ions, and sensitive to divalent cation chelators such as EDTA and EGTA (Smith & Chisholm, 1992; Marques & Barracco, 2000), and a few exception to these features have been reported (Imai et al., 1994; Maheswari et al., 1997; Kondo et al., 1992). Dialysis experiment performed in this study against cation-free TBS demonstrated that the HA activity in the crab serum is dependent on divalent cations such as Ca$^{2+}$, Sr$^{2+}$ or Mg$^{2+}$, since the activity was not detected in the absence of these divalent cations. Moreover, the activity was recovered upon addition of these cations, but complete recovery was achieved only with CaCl$_2$, suggesting that the serum HA activity exhibits requirement for divalent cations, with a preference for Ca$^{2+}$. Similar results were obtained from dialysis experiment performed with EDTA, thereby indicating that the serum
HA activity is reversibly sensitive to EDTA, a divalent cation chelator. Furthermore, serial dilution experiments performed with both EDTA and EGTA showed that the serum HA activity is sensitive to EDTA, whereas it is only partially sensitive to EGTA. Further experiments with various concentrations of different divalent cations demonstrated that HA activity increased in the presence of especially CaCl₂ or SrCl₂ in a dose-dependent manner, with a maximal activity detectable at 50 mM.

In hemagglutination-inhibition tests performed in this study, many carbohydrates encompassing several diverse unrelated monosaccharides and their derivatives as well as di- and oligo-saccharides inhibited the serum agglutinating activity. Furthermore, all the three acetylated hexosamines (GlcNAc, GalNAc and ManNAc), but not their hexose and hexosamine counterparts tested, consistently inhibited the HA activity of crab serum, thereby suggesting the affinity of serum agglutinins for acetylated aminosugars. It is also notable that the two sialic acids, Neu5Ac and Neu5Gc, tested were not inhibitory even up to concentrations of 40 and 20 mM, respectively. By contrast, Mercy & Ravindranath (1992) demonstrated the inhibitory activity (at 0.6 mM) of Neu5Gc as well as inability of all the three acetylated hexosamines to inhibit the serum HA activity of S. serrata. The exact reason for these discrepancies between the two studies is not clear at present. Further studies with polysaccharides indicated that only laminarin and mannan weakly inhibited the serum agglutinating activity, while free glucose and mannose were non-inhibitory, indicating that the agglutinin
molecules in crab serum tend to exhibit affinity for extended structures particularly for polysaccharides with β-linked hexoses.

Further inhibition tests were performed with glycoproteins comprising of eight sialo- and asialo-glycoconjugates, whose oligosaccharide side chains, as reported earlier (Schauer, 1982; Tsuji & Osawa, 1986; Corradi Da Silva et al., 1995), contain predominantly terminal Neu5Gc (thyroglobulin and PSM), Neu5Ac (fetuin, BSM, and de-O-acetylated BSM), galactose (asialo-fetuin), GalNAc (asialo-BSM), and GlcNAc (ovalbumin). In this inhibition assay, the serum agglutinin appears to show higher affinity for sialoglycoproteins terminated with Neu5Ac (fetuin and BSM) or Neu5Gc (PSM and thyroglobulin) as well as desialylated glycoproteins, namely, asialo-fetuin and asialo-BSM terminated with Gal and GalNAc, respectively. These observations suggest that the serum agglutinins recognize two types of sialic acids (Neu5Ac or Neu5Gc) which is apparent in inhibition studies with glycoproteins, but not with free sialic acids. Thus, all the results obtained from the inhibitory effects of various carbohydrates and glycoproteins taken together clearly indicate that the agglutinins present in the serum of S. serrata interact with a wide range of carbohydrates including acetylated hexosamines, acetylated or non-acetylated sialic acids and several other carbohydrates, and their preference for a specific carbohydrate structure, therefore, could not be ascertained. However, these findings in turn strongly suggest the natural occurrence of multiple agglutinins in the serum of this crab.
It is interesting to note that the serum agglutinated a variety of bacteria including Gram +ve and -ve types and the species of *Vibrio* tested are known to be the most frequent opportunistic pathogens of aquatic crustaceans (Egidius, 1987). The ability of the serum of *S. serrata* to agglutinate the bacteria, particularly the potential pathogens, implicates a possible involvement of the humoral agglutinins in host defense response.

2.0 ISOLATION AND CHARACTERISATION OF MULTIPLE AGGLUTININS FROM THE SERUM OF *SCYLLA SERRATA*

In this study, three naturally occurring agglutinins were successfully isolated at high purity from the serum of *S. serrata* by affinity chromatography using thyroglobulin-Sepharose 4B (Agglutinin 1), BSM-Sepharose 4B (Agglutinin 2), and GlcNAc-Sepharose 6B (Agglutinin 3) as affinity matrices and EGTA or GlcNAc for elution.

It may be recalled that analysis of serological properties of the whole serum of *S. serrata* revealed its reactivity with high titers against rabbit, mouse, human B, rat, and buffalo RBC. Similarly, all the three isolated serum agglutinins agglutinated these five RBC types, but with significant differences in the HA titers. Agglutinins 1 and 2 strongly reacted with human B RBC, whereas Agglutinin 3 gave the highest titer for mouse RBC, thereby demonstrating that these three agglutinins not only exhibit preference but also cross-reactivity in binding to surface of different types of foreign cells with varying affinity. At the same time, the whole serum of this crab was found to agglutinate rabbit and rat RBC with high HA titers, but none of the three
isolated serum agglutinins expressed such a reactivity against the two RBC types. In this respect, these three agglutinins appear to differ from the two agglutinins previously isolated from the serum of S. serrata (Chattopadhyay & Chatterjee, 1993; Mercy & Ravindranath, 1993). Nevertheless, the discrepancy observed in the present study with reference to reactivity of whole serum and the isolated agglutinins against the murine RBC tested is attributed to the fact that the dilute serum, before passing through the affinity column, had HA titers of 8 and 4 for rabbit and rat RBC, respectively, and almost the same levels of these activities were detectable in the serum effluent collected from each column. These findings clearly suggest that the serum of S. serrata does contain at least one more agglutinin with a strong reactivity for rabbit and rat RBC, and that it never adsorbed to any of the three affinity gel matrices used in this study.

Animal lectins have been broadly classified into five major types (C, I, S, and P types, and pentraxins) based on the nature of their carbohydrate ligands, the biological processes in which they participate, subcellular localisation, and dependence on metal ions (Drickamer & Taylor, 1993; Gabius, 1997). The serum agglutinins of most crustaceans are known to be dependent upon divalent cations, usually Ca\(^{2+}\), and they are reversibly or irreversibly sensitive to divalent cation chelators such as EDTA and EGTA (Smith & Chisholm, 1992; Marques & Barracco, 2000). In a few crustaceans, the activity of serum agglutinins does not require divalent cations (Kondo et al., 1992; Imai et al., 1994; Maheswari et al., 1997), and it is rarely insensitive to the divalent cation chelators (Maheswari et al., 1997), a feature quite uncommon among
crustacean agglutinins. In the present study, Agglutinins 1 and 2 appear to be C-type agglutinins, since they require metal ions (Ca\textsuperscript{2+}) for their activity, and additionally they are sensitive to EDTA and EGTA. By contrast, the activity of Agglutinin 3 is neither dependent on Ca\textsuperscript{2+} nor is it sensitive to the divalent cation chelators, the former feature being a characteristic of S-type agglutinins, and thus Agglutinin 3 is distinct from Agglutinins 1 and 2. It also becomes apparent that the persistence of some agglutinating activity detectable in EGTA-treated whole serum of this crab is due to the activity contributed by Agglutinin 3. All these findings clearly demonstrate the existence of multiple agglutinins in the serum of *S. serrata* with a differential requirement for Ca\textsuperscript{2+} and sensitivity to divalent cation chelators, and the functional significance of this feature is presently unknown.

In native PAGE, each isolated agglutinin appeared as a single protein band on staining the gel with Coomassie brilliant blue or silver nitrate. The latter is known to detect as low as 0.2 ng protein per band (Ansorge, 1985), thereby demonstrating that each agglutinin was purified to electrophoretic homogeneity by a single-step affinity chromatography on empirically selected gel matrices. Furthermore, the protein bands of these agglutinins corresponded with faint bands visible in the concurrent electrophoresis of the whole serum of *S. serrata*, indicating a typical enrichment or concentrating effect of affinity chromatography. Besides, the electropherogram showed distinct differences in velocity of migration among Agglutinins 1, 2 and 3 examined in active forms and whose molecular weights were estimated to be 353, 477 and 639 kDa, respectively. These are considered to be approximate values, since the
separation in native PAGE involves both the total net charge mass and molecular size of the proteins, and further analyses of these agglutinins in gel filtration, SDS-PAGE and analytical isoelectric focussing would provide valuable information on their molecular size and subunit structure. Despite this limitation, these three isolated agglutinins, each with high molecular mass, appear to differ from those (4.8 to 5.0, 55 and 70 kDa) reported by previous investigations with S. serrata (Chattopadhyay & Chatterjee, 1993; Mercy & Ravindranath, 1993; Kongtawelert, 1998). However, single or multiple agglutinins with high molecular weights (even up to 700 kDa) are known to occur in the serum of crustaceans (Marques & Barracco, 2000).

A perusal of previous studies with singular type of agglutinins isolated from the sera of several crustacean species reveals their specificity for a wide range of carbohydrates, such as ribose, galactosyl or glucosyl residues, fucose, lactose, and sialic acids, namely, Neu5Ac and Neu5Gc (Smith & Chisholm, 1992; Marques & Barracco, 2000). In similar studies, serum agglutinins with an apparent or exquisite specificity for acetylated hexosamines have been isolated from the serum of many crustaceans including the prawns and shrimps (Ratanapo & Chulavatnatol, 1990; Vargas-Albores et al., 1993; Cominetti et al., 2002; Maheswari et al., 2002), and crabs (Cassels et al., 1994; Murali et al., 1999). On the other hand, none of the previous studies dealing with multiple agglutinins elucidated the nominal carbohydrate-binding specificities of all the agglutinin fractions isolated from the crustacean sera, and an exception being the two hetero-agglutinins isolated from the serum of the lobster H. americanus (VanderWall et al., 1981; Table III).
In the present study, extensive inhibition assays performed with several carbohydrates and glycoproteins gave an insight into the carbohydrate-binding properties of all the three agglutinins isolated from the serum of *S. serrata*. The assays with carbohydrates revealed many salient features of agglutinin-ligand interactions: (1) The three isolated agglutinins showed two distinct differences in their nominal specificities; Agglutinins 1 and 2 expressed the highest affinity for pNP-α-D-glucopyranoside, whereas Agglutinin 3 for GlcNAc. (2) Both Agglutinins 1 and 2 were found to interact with many diverse and often overlapping carbohydrates with varying affinity. Strikingly, the interaction of Agglutinin 3 was restricted towards a few carbohydrates, especially acetylated hexosamines which were also recognized by two other agglutinins. (3) Agglutinins 1 and 2 interacted with sialic acids, but with a distinct qualitative difference; Agglutinin 1 recognized Neu5Gc and not Neu5Ac, and *vice versa* for Agglutinin 2. By contrast, Agglutinin 3 did not bind both types of sialic acids. (4) Agglutinins 1 and 2 reacted indiscriminately with all the eight glycosides tested, while Agglutinin 3 never interacted with them. (5) Finally, Agglutinins 1 and 2 exhibited a clear tendency for extended carbohydrate structures such as di- and oligo-saccharides, but this feature was lacking with Agglutinin 3.

Further inhibition studies demonstrated the ability of Agglutinins 1 and 2 to react with different types of polysaccharides including laminarin, mannann, and zymosan (containing glucans and mannans) which are the major components of fungal cell wall. As observed with free carbohydrates, Agglutinin 3 showed a restricted interaction with polysaccharides by binding
only to laminarin. Although, the variations recorded in the reactivity of the three agglutinins appears to be unrelated to the type of carbohydrates or nature of glycosidic linkages associated with the polysaccharides tested, these findings, suggest the ability of all the three agglutinins to interact with fungal cell wall components. On the other hand, these agglutinins exhibited significant differences in binding to various glycoproteins. Agglutinins 1, 2 and 3 expressed preference for specific glycoconjugates, namely, thyroglobulin and PSM, BSM, and ovalbumin, respectively. The ability of individual agglutinin of *S. serrata* to recognize and bind to the specific glycoprotein with high affinity is clearly correlated with the presence of appropriate terminal carbohydrate residues in such glycoproteins for which the agglutinin molecules possess the complementary binding site (Schauer, 1982; Tsuji & Osawa, 1986; Corradi Da Silva *et al.*, 1995), which are in accordance with the findings from inhibition assays performed with free carbohydrates. Besides, Agglutinins 1 and 2 bound with a lesser affinity to all other glycoproteins tested, whereas a restriction in the reactivity of Agglutinin 3 was consistently evident by its binding to only two other glycoconjugates. It is pertinent to note that Agglutinin 1 with specificity for NeuGc and thyroglobulin, and Agglutinin 2 expressing high affinity for BSM appear to resemble, respectively, the agglutinins previously isolated from the serum of *S. serrata* by Mercy and Ravindranath (1993) and Kongtawelert (1998).

All the findings from inhibition assays taken together clearly demonstrate that the multiple agglutinins in the serum of *S. serrata* possess unique mixed binding properties: reactivity with diverse carbohydrates
(Agglutinins 1 and 2), restricted specificity for certain carbohydrate ligands (Agglutinin 3), overlapping feature of interaction with many disparate carbohydrate moieties (Agglutinins 1, 2 and 3), and ability to bind glycoconjugates through recognition of appropriate sugar residues (Agglutinins 1, 2 and 3). However, it is currently unknown whether each agglutinin contains a single promiscuous combining site or different sites capable of binding to related and unrelated ligand structure.

It is also notable that carbohydrate-binding properties of one of the three agglutinins (Agglutinin 1) isolated in the present study from the serum of S. serrata showed similarity with the serum agglutinin previously isolated by Mercy & Ravindranath (1993). In comparison with this as well as two other earlier reports (Chattopadhyay & Chatterjee, 1993; Kongtawelert, 1998), the three agglutinins isolated in this study are distinct with respect to their apparent native molecular weights and carbohydrate-binding specificities. These discrepancies may be partly due to the different strategies adopted in affinity chromatography employed for isolation of serum agglutinins of S. serrata. Nevertheless, the present as well as previous studies strongly indicate that more than three multiple agglutinins naturally occur in the serum of S. serrata.

Bacteria possess numerous potential surface structures such as peptidoglycans, teichoic acid, LPS, and capsular materials that are accessible for interaction by agglutinins. It is known that naturally occurring agglutinins isolated from the sera of many crustaceans interact with LPS from various
bacterial species as well as cause agglutination of a variety of Gram +ve and -ve bacteria (Ratanapo & Chulavatnatol, 1992; Chattopadhyay & Chatterjee, 1993; Kopáček et al., 1993; Vargas-Albores et al., 1993; Cassels et al., 1994; Fragkiadakis & Stratakis, 1995, 1997; Vázquez et al., 1996; Murali et al., 1999; Maheswari et al., 2002). It is well known that Vibriosis is one of the most serious diseases of penaeid shrimps as well as other cultivable crustaceans, and several species of Vibrio, including V. anguillarum, V. alginolyticus, V. parahemolyticus and V. mimicus, are the most frequent opportunistic bacterial pathogens of cultured crustaceans (Takahashi et al., 1985; Egidius, 1987; Lightner et al., 1992; Limsuwan, 1994). In the present study, Agglutinins 1 and 2 were susceptible to inhibition by LPS from diverse bacterial species, and they were able to agglutinate several species of Gram +ve and -ve bacteria, and exhibited strong agglutination reaction particularly against all the six Vibrio species examined. By contrast, Agglutinin 3, for unknown reason, did not apparently bind to any of the LPS types tested, but it agglutinated, as observed with Agglutinins 1 and 2, all the bacterial species tested in this study. In this respect, the activity of these multiple agglutinins differ from an agglutinin isolated previously by Chattopadhyay & Chatterjee (1993) which was shown to strongly react against Gram +ve bacteria. However, the exact site of LPS or other bacterial cell wall components involved in the interaction of the serum agglutinins is not clear at present. However, acetylated aminosugars (Neu5Ac, GlcNAc) as well as other types of carbohydrate moieties have been detected in LPS coat or peptidoglycan layer of bacteria (Lüderitz et al., 1968; Schauer, 1982; Slifkin & Doyle, 1990; Gamian et al., 1994; Charland ct al., 1995). Thus, it is conjectured that the multiple
humoral agglutinins of *S. serrata* with an ability to recognize diverse carbohydrate structures could directly interact with different types of microbes including fungi and bacteria, thereby serving as non-self recognition molecules of the humoral immune system and possibly mediating opsonic functions in this crab.

3.0 FUNCTIONAL ANALYSES OF ISOLATED MULTIPLE SERUM AGGLUTININS IN CELLULAR IMMUNE RESPONSES OF *SCYLLA SERRATA*

Multiple agglutinins have been previously isolated from the body fluids of several invertebrate groups such as arthropods, molluscs, echinoderms and prochordates. But none of these studies, with one exception, attempted to examine the role of the multiple agglutinins in immuno-defense functions. The report by Wilson et al. (1999) represents the only unique study involving analyses of opsonic properties of four hetero-agglutinins isolated from the serum of the cockroach, *Blaberus discoidalis*. This recent experimental investigation elegantly demonstrated *in vitro* the ability of all the four humoral agglutinins, with defined nominal carbohydrate binding specificities, to induce the phagocytic response of cockroach hemocytes against baker's yeast and two bacterial species. In deed, the findings of this study prompted us to investigate the role of three agglutinins isolated from the serum of *S. serrata* in hemocyte-mediated cellular immune responses.

The present study examines *in vitro* the role of multiple agglutinins on the immune responses of hemocytes of *S. serrata*, and various aspects of
this experimental investigation as well as reasons for their consideration have been summarized here.

1) The response of hemocytes were examined *in vitro*, because this test system permits desirable experimental manipulations and conclusive assessment of the hemocytic functions, as opposed to *in vivo* state which not only lacks these advantages, but also has inherent complexity.

2) Three types of hemocytic responses quantitatively assayed in this study include attachment or adherence of particulate foreign materials on to the surface of hemocytes, subsequent phagocytic uptake of the same types of foreign targets, and finally encapsulation response of hemocytes against larger size particulate targets. These three types of hemocytic responses were chosen for this study, since all of them are easily observable, and thus any change in these cellular responses with test substances can be unambiguously detected. Besides, attachment of foreign targets on to hemocyte surface implicates receptor-mediated non-self recognition capability of hemocytes, whereas phagocytic and encapsulation responses of hemocytes, which are related to the size of foreign objects dealt by these cells, represent two distinct and important manifestations of hemocyte-mediated cellular immune reactions.

3) Although the primary objective of this investigation was to examine the modulatory effects of multiple agglutinins isolated from the serum of *S. serrata*, the whole serum, from where these agglutinins were isolated, was first tested in identical experimental conditions in order to gain an insight into the performance of the source material.
4) A panel of three kinds of foreign particulate materials, encompassing five types, have been used in the present study to analyse attachment and phagocytic responses of hemocytes of *S. serrata*, and these materials include three mammalian (human B, mouse and ox) RBC types, yeast (*Saccharomyces cerevisiae*), and bacteria (*Vibrio fluvialis*). The agglutination profiles of both the whole serum and all the three isolated serum agglutinins indicated moderate to strong interaction of serum agglutinins with these foreign targets, except ox RBC. Despite this fact, the latter cell type was also included in the panel of test particles in assays with the whole serum for a better understanding of functional significance of serum agglutinins. It is also notable that the yeast was found to be agglutinated (data not given previously) by whole serum (titer = 4) as well as the three isolated serum agglutinins (+), and HA activities of all these samples were inhibited by zymosan, a major component of yeast cell wall.

5) In studies using FITC-labelled bacteria, a difficulty was encountered in determining the attachment of bacteria to hemocyte surface, and this was due to an ambiguity in enumerating the actual number of such bacteria associated with the surface of each hemocyte, an essential prerequisite to score the positively responding hemocytes in monolayers. Therefore, the analysis with bacteria was inevitably confined to assays of phagocytic response of hemocytes.

6) All the test samples were used at subagglutinating concentrations, as determined by their HA titers or reactivity against various foreign targets employed in the assays of attachment and phagocytic responses of the hemocytes. This protocol was consistently adopted in order to avoid clumping of the targets in the *in vitro* test system, which would otherwise impede the phagocytic response of hemocytes *in vitro*. 
7) Finally, chromatographic gel beads (Sepharose CL-6B) were chosen for quantitative assays of encapsulation response of hemocytes under various test conditions, because of their large size and proven usefulness, and they can be obtained without or with specific charged groups on their surfaces.

The hemolymph of *S. serrata* coagulates rapidly after extravasation, and the process of coagulation essentially involves degranulation and lysis of hemocytes. Therefore, cysteine or NEM was used as anticoagulant as a prerequisite to obtain hemocytes in native form that would eventually enable *in vitro* analyses of phagocytic and encapsulation responses of the crab hemocytes (Nalini, 2002). In the present study, over 90% of the hemocytes obtained using these anticoagulants remained viable, a feature conducive for functional analyses of hemocytes *in vitro*. Moreover, the hemocytes harvested using cysteine, but not NEM, were able to form monolayers on glass surfaces, and this hemocytic behaviour was considered suitable for analysis of their *in vitro* phagocytic activity. On the other hand, the hemocytes obtained with NEM were more stable in suspension, and this protocol was, therefore, followed to obtain hemocytes for assays of their encapsulation response *in vitro*.

In a series of experimental studies performed in the present study, small proportions of *S. serrata* hemocytes in control monolayers always showed attachment and phagocytosis of all the five types of particulate targets tested. These basal responses may be considered to be due to non-specific interaction of hemocytes with the targets. By contrast, the hemocytes consistently
expressed significantly higher and varying levels of attachment and phagocytic activity against serum-pretreated human B and mouse RBC, yeast and phagocytosis of serum-pretreated bacteria, but not with ox RBC. It is pertinent to note from these tests that the proportions of phagocytic hemocytes were invariably much lesser than those showing intense attachment with serum-pretreated RBC or yeast. This discrepancy may be due to a slow phagocytic process in crab hemocytes particularly against larger size targets, since the hemocytes ingested rapidly bacteria than other targets used in this study. Nevertheless, these findings clearly suggested that the serum of S. serrata contains soluble factors capable of binding with varying affinity to the surface of targets bearing appropriate complementary structures. Such binding appeared to have facilitated hemocytes to recognize and interact with the targets. It becomes, therefore, apparent that these factors serve as non-self recognition molecules as well as an opsonic function by stimulating the phagocytic activity of the crab hemocytes. Conversely, the lack of significant interaction between hemocytes and ox RBC may be attributed to inability of such soluble serum factors to bind to surface of this RBC type. All these observations, corroborated by findings from agglutination profiles, appear to implicate the opsonic role of serum agglutinins in the hemocytic responses described above.

Further investigations were, therefore, extended towards analyses of three isolated agglutinins under identical test conditions employed for whole serum, except that ox RBC were not used owing to poor hemocytic responses. In these experimental studies, as observed with whole serum, the hemocytes
were found to express higher levels of interaction (by way of attachment) and phagocytic responses against human B RBC pretreated with subagglutinating concentrations of Agglutinin 1 or 2. Similar results, with more pronounced responses, were also obtained with mouse RBC pretreated with a subagglutinating concentration of Agglutinin 3. Moreover, the hemocytes of S. serrata were found to express consistently enhanced (2 to 5 fold) attachment and phagocytic uptake of yeast or bacteria pretreated with Agglutinin 1, 2 or 3. It is important to note from these in vitro experimental studies that the promoting effects of the three isolated serum agglutinins were prominent even at 0.7 to 88 μg.ml⁻¹, which were 2 to 124-times lesser than their estimated physiological concentrations, and it is, therefore, anticipated that the multiple serum agglutinins could accomplish their opsonic effects more effectively in vivo. All these findings unambiguously demonstrate that the three agglutinins isolated from the serum of S. serrata facilitate non-self recognition of a wide range of foreign materials as well as mediate their opsonophagocytosis by the crab hemocytes.

In another experimental study, the hemocytes of S. serrata were found to encapsulate preferentially positively-charged DEAE-Sepharose beads suspended in buffer alone, but never reacted with neutral and negatively-charged (CM-Sepharose) beads under identical test conditions. These observations suggest that the responding hemocytes possess net negative charges on their surface that enable them to attach selectively and directly to positively-charged beads. This response of hemocytes was significantly enhanced in the presence of plasma, indicating the occurrence of encapsulation-promoting factors in the cell-free hemolymph of S. serrata, a
finding contradictory to the previous reports with other crustacean species (Söderhäll et al., 1984; Persson et al., 1987; Kobayashi et al., 1990; Amirante, 1992; Battistella et al., 1996 a, b). On the other hand, the presence of serum did not promote the encapsulation response of crab hemocytes against DEAE Sepharose beads, but rather it tend to inhibit this response. This interesting observation suggests that the crab serum probably contains encapsulation-suppressing factors, and it is reasonable to expect that the serum could have derived such inhibitory factors from hemocytes that are well known to undergo lysis during the process of hemolymph coagulation (Nalini, 2002).

In order to elucidate the role of serum agglutinins in cellular encapsulation response, all the three isolated serum agglutinins were subsequently examined for their ability to modulate this hemocytic response. Surprisingly, it was found that the presence of Agglutinin 1 strongly suppressed the encapsulation response of hemocytes against DEAE-Sepharose beads. By contrast, two other agglutinins (Agglutinins 2 and 3) significantly enhanced this hemocytic response. It is notable that none of the isolated serum agglutinins promoted the encapsulation response of hemocytes against neutral or CM Sepharose beads. Although the actual mechanisms by which these agglutinins accomplish differential effects in encapsulation response of hemocytes are not clear at present. However, these observations, nevertheless, reveal two important functional features of the three serum agglutinins upon comparison of their effects with those recorded for plasma and serum of S. serrata: (1) Agglutinins 2 and 3 with encapsulation-promoting property are likely to be true humoral agglutinins naturally occurring in plasma, and (2) Agglutinin 1 with a unique encapsulation-suppressing effect appears to be
localized intracellularly within the hemocytes. In deed, the presence of agglutinating activity in lysates of *S. serrata* hemocytes has been detected in a previous study performed in this laboratory (Nalini, 2002), but the identity of this hemocytic agglutinin remains to be determined. Since hemocytes of crustaceans have been shown to degranulate and release their contents consequent to interaction with non-self materials (Johansson & Söderhäll, 1985; Söderhäll et al., 1986), it is plausible that Agglutinin 1 could be released under such conditions, thereby it becomes accessible for interaction with targets or hemocytes. In such events, it is envisaged that Agglutinin 1 can modulate the encapsulation-promoting activities of Agglutinins 2 and 3 already present in plasma. Clearly, further investigations should be directed towards elucidating the mechanism of interactions among the multiple serum agglutinins of *S. serrata* during the encapsulation response of hemocytes. Moreover, these agglutinins may not be the only opsonic factor in the serum of *S. serrata*, since the presence of other types of humoral molecules with similar functional features in the serum of this crab cannot be ruled out.

In conclusion, this study unambiguously demonstrates, *for the first time in a crustacean*, the multiple serum agglutinins not only play vital role in recognition of a wide range of foreign materials including microorganisms by hemocytes but also eventually serve opsonic functions, with an apparent equi-potency, by facilitating hemocyte-mediated cellular immune responses, namely, phagocytosis and encapsulation. Interestingly, they also appear to modulate or regulate encapsulation response of the crab hemocytes, *a finding first of its kind in an invertebrate*. Finally, the findings of the present study,
by revealing the efficacy of multiple serum agglutinins, emphasize the importance of these agglutinins in innate immunity, and thus obviate the need for circulating antibodies in crustaceans as well as other coelomate invertebrates.