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Hemagglutinin and Hemolysin in Korean Ascidians

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Two kinds of humoral factors were observed in 2 orders, 7 families, 10 genera, and 15 different species of Korean ascidians. They are the naturally occuring hemagglutinins and/or hemolysins against human erythrocytes A, B, and O. All but two species showed aggregative activity, although there were considerable variations in titer. The weak agglutinating and lytic activities were increased in the presence of Ca^{++}. Much higher activities of agglutination and/or lysis were shown in the hemolymph than extracts from tissues, and a higher response was shown in adults than in juveniles. No distinct differences from collected locations were observed. The hemolymph of *Ciona intestinalis* showed a strong hemolytic (cytotoxic) and weak agglutining capacities. In addition, hemolymph of *Styela plicata* and *Styela clava clava* also showed hemoagglutining and hemolytic activities. *Botryllus tuberatus* had hemagglutining and weak lytic activities. Other species showed only hemagglutining activity. These agglutining activities are probably responsible for carbohydrate recognition in solitary or colonial ascidian. The lytic activity is probably responsible for antibacterial defense and nonfusion reactions between allogeneic colonial ascidians, especially the genus of *Botryllus*. The occurrences of humoral factors in ascidians were independent of their geographic distributions.

Ascidians are sessile marine invertebrates with a world-wide distribution. Most of them usually live in shallow water by attaching to rock, shells, and pilings. Some are found in mud and sand and some are cultivated. They comprise about 2,300 species and live in solitary or as colonies. The ascidian fauna of Korea is moderately abundant, as both the colony and solitary form, with about 80 species in 11 families (Rho and Huh, 1984). Although many previous works have been done on faunistic and ecological accounts of the Korean ascidians (Rho, 1975, 1995; Rho and Huh, 1984; Rho and Lee, 1989, 1991; Rho et al., 1996), at present, there is lack of data concerning the humoral factors (lectins) in Korean ascidian species.

Among invertebrates, ascidians are considered as the primitive members of phylum Chordata. They are protochordata occupying the phylogenetic position leading to vertebrates based on their evolution process of humoral, cellular and immuno-defense systems. Several humoral factors (e.g., hemagglutinins, hemolysins, opsonins, antimicrobial substance, factor of allorecognition, and cytokines) and hemocytes (e.g., macrophages and natural killer cells) are expressed in ascidian hemolymph and tissues which are not involved in immune defense but play a role in budding for reproduction (Fuke and Sugai, 1972; Kawamura et al., 1991). Various kinds of humoral factors and hemocytes in ascidians have been well reviewed especially with respect to their functions by Saito (1996). Hemocytes in ascidians also have been well reviewed by Parrinello (1996). Lectins (humoral factors) are generally considered to be naturally occurring proteins which can selectively bind to specific carbohydrates. They are sometimes produced when injured or during budding. They are present in the hemolymph (soluble-lectin), hemocytes (cellular-lectin), and cell wall of ascidian. Carbohydrate recognition of lectins is involved in agglutining of animal erythrocytes (hemagglutin), lysis of erythrocytes (hemolysin), adhesion between cell to cell, egg and sperm, and adhesion of cell to the extracellular matrix. The roles of ascidian humoral factors are important in their defense system, fertilization, and budding. The hemolymph of many ascidians has been tested and found that it has agglutinating activity for vertebrate erythrocytes. Most of these agglutinins are heat labile, and can be digested by proteolytic enzymes. Antimicrobial or antiviral substances are also one of the most important humoral factors in their defense mechanisms. Two antimicrobial substances, halocyamine A and B were isolated from the hemocytes of a solitary ascidian, *Halocynthia roretzi* (Azumi et al., 1990). Both antimicrobial substances inhibited the growth of some gram-positive bacteria and yeast. Halocyamine A inhibited the *in vitro* growth of fish RNA viruses and it has cytotoxic activity against some cultured mammalian cells.

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Table 1. Comparative activity of humoral factors in Korean ascidians

Species	Hemagglutinin			Hemolysin		
	O (fuc)	A (galNAc)	B (gal)	O (fuc)	A (galNAc)	B (gal)
Amaroucium pliciferum	-	-	-	-	-	-
Didemmum sp.	++++[1]	+++++	+++++	-	-	-
Chelyosoma siboga	++	++	++	-	-	-
Botryllus tuberatus	+++	+++	+++	+	+	+
Ciona intestinalis	++	++	++	+++++	+++++	+++++
Dendrodoa aggregata	+	+	+	-	-	-
Styela plicata	++	++++	++	+++	+++	+++
S. clava clava	++	++++	++	+++	+++	+++
Pyura sacciformis	++	++	++	-	-	-
Herdmania mirabilis	++	++	++	-	-	-
Halocynthia roretzi	++	++	++	-	-	-
H. aurantium	-	-	-	-	-	-
H. hilgendorfi igaboja	++++	++++	++++	-	-	-
H. hilgendorfi ritteri	+++	+++	+++	-	-	-
H. cactus	+++	+++	+++	-	-	-

[1]Degree of activity : +++++ > ++++ > +++ > ++ > + > -
fuc: fucose, galNAc : N-acetyl-galactosamine, gal: galactose

In this present study, we performed preliminary comparative studies which deals with the humoral factors from 15 Korean ascidian species. This study may provides valuable information in elucidating the molecular phylogenic relationship between genera and their defense functions.

Materials and Methods

Collection and preparation of the samples

One hundred seven ascidians were collected from five localities (Teokdong, Isudo, Cheondo, Tangsa, Hungnam) around the southern and eastern coast of Korea by SCUBA divers. Specimens were maintained in seawater for several hours and kept in dry ice for hemagglutinins and hemolysins test. Hemolymph was collected from the blanchial or atrial siphon and the gill slit of solitary ascidians by gently squeezing colonial ascidians. The stomach, esophagus, heart, and gonad of solitary ascidian of individual animal was mixed with an equal volume of phosphate buffered saline (PBS), minced with scissors and homogenized. The samples were centrifuged at 6,000xg for 30 min at 4℃. The supernatant was used for the hemagglutining test.

Hemagglutination and hemolysis assay

Four percent (v/v suspension) of human A, B, and O erythrocytes were treated with 1% trypsin (Gibco 1 : 250) for 60 min at 37℃ and washed 3 times in saline. Quantitative hemagglutination or lysis of each blood type was performed at room temperature for 5-30 min. A 150 μl extract an of ascidian was mixed with an equal volume of human erythrocyte suspension in PBS. The specificity of the hemagglutining and hemolytic activities were determined with human A, B, and O erythrocytes whose sugar specificity were N-acetyl-galactosamine, galactose, and fucose, respectively, using PBS or PBS-Ca^{++}.

Identification

For identification, ascidians were relaxed by menthol powders for several hours and then fixed in solution of 4-5% formaldehyde and water. To observe the internal structure, they were kept in a solution of 1% chromic acid and 50% acetic acid in a 1:10 ratio for a day. They were placed in 1% chromic acid for a few hours before dissection. Identification and classification scheme used in this study were based on Ven Name (1945), Rho (1975), and Kott (1985).

Results

Naturally occurring humoral factors, hemagglutinins and hemolysins were found to be widely distributed in 7 families of Korean ascidians tested. All but two species possessed detectable humoral factors in hemolymph; the hemagglutinins reacted with all of human A, B, and O erythrocytes. There were considerable variations in titer. However, erythrocyte sugar carbohydrate specificity of individual specimens and haemagglutining titer were not varied and depended on the erythrocyte type A (N-acetyl-galactosamine), B (galactose), and O (fucose) (Table 1). Generally, hemagglutining activity increased slightly with the trypsin-treated erythrocytes compared to the untreated erythrocytes. Hemagglutining and hemolytic activities in hemolymph increased 5-10 folds compared to those in tissue extracts, and higher activities were shown in adults than in juveniles.

The divalent cation was not required for activity, although agglutining or lytic activity was slightly increased in the presence of Ca^{++}.

Table 2. Humoral factors in ascidians of various locations in the world

Species	Humoral	Location	Form	Source	Reference
Order Enterogona					
Suborder Aplousobranchia					
Family Polyclinidae					
Amaroucium pliciferum	N	South Korea	C	HL	Present study
Aplidium australiensis	A	South Australlia	C	PL	Coombe et al., 1984
Family Didemnidae					
Didemnum patulum	A	South Australlia	C	PL	Coombe et al., 1984
D. candidum	A, L	USA	C	PL	Vasta and Marcalonis, 1986
Didemnum sp.	A	South Korea	C	HL	Present study
Diplosoma sp.	N	South Australlia			Coombe et al., 1984
Family Polycitoridae					
Atapozoa fantsiana	A	South Australlia	C	PL	Coombe et al., 1984
Family Holozoidae					
Sycozoa tenuicaulis	N	South Australlia			Coombe et al., 1984
Suborder Phlebobranchia					
Family Clavelinidae					
Podoclavella cylindrica	N	South Australlia			Coombe et al., 1984
Family Cionidae					
Ciona intestinalis	A, L	South Korea	S	HL	Present study
	A, L		S	PL	Wright, 1974; Coombe et al., 1984 Wright and Cooper, 1975; Parrinello and Patricolo, 1975;
	A, L	Italy		HC	Parrinello et al., 1993
Family Ascidiidae					
Ascidia ceratodes	A		S	PL	Tyler, 1946
A. malaca	A		S	PL	Parrinello and Patricolo, 1975; Parrinello and Canicatti, 1982; Yokosawa et al., 1986 Cantacuzene, 1919
				HC	Parrinello and Arizza, 1988
A. mentula	A	South Australlia	S	PL	Coombe et al., 1984
A. thompsoni	A		S	PL	Parrinello and Patricolo, 1975;
Phallusia mammillata				HL	Parinello and Canicatta, 1983 Renwrantz and Uhlenbock, 1974
	A, L			HC	Parrinello and Arizza, 1989; Cammarata et al., 1993
		Southern France			Bretting & Renwrantz, 1974
P, despressiuscula	A	South Australlia	S	PL	Coombe et al., 1984
Family Rhodosomatidae					
Chelyosoma siboga	A	South Korea			Present study
Order Pleurogona					
Suborder Stolidobranchia					
Family Botryllidae					
Botryllus tuberatus	A, L	South Korea	C	HL	Present study
Botrylloides leachii	A	South Australlia	C	PL	Coombe et al., 1982; Coombe et al., 1984
B. mabnicoecus	A	South Australlia	C	PL	Coombe et al., 1984
B. primigenas					
Family Styelidae					
Polyandrocarpa misakiensis		Japan	C	EP	Suzuki et al., 1990; Kawamura et al., 1991
Stolonica australis	A	South Australlia	C	PL	Coombe et al., 1984
Cemidocarpa etheridgii	A	South Australlia	S	PL	Coombe et al., 1984
Polycarpa obtecta	A	South Australlia	S	PL	Coombe et al., 1984
Polycarpa pedunculata	N	South Australlia			Coombe et al., 1984
P. papillata	A	South Australlia	S	PL	Coombe et al., 1984
Dendrodoa aggregata	A	South Korea	S	HL	Present study
Styela plicata	A, L	South Korea	S	HL	Present study
	A		S	PL	Fuke and Sugai, 1972
S. clava clava	A, L	South Korea	S	HL	present
S. clava	A	Califonia	S	PL	Wright and Cooper, 1984
Family Pyuridae					
Halocynthia cactus	A	South Korea	S	HL	Present study
Halocynthia hilgendorfi	A		S	PL	Fuke and Sugai, 1972
H. hilgendorfi ritteri	A	South Korea	S	HL	Present study
H. hilgendorfi igaboja	A	South Korea	S	HL	Present study
H. pyriformis	A		S	PL	Anderson and Good, 1975; Form et al., 1979; Vasta and Marchalonis, 1983
H. papillosa	A		S	PL	Bretting and Renwrantz, 1973
H. aurantium	N		S	HL	Present study
H. roretzi	A	South Korea	S	HL	Present study
	A	Japan Hokkaido	S	PL	Yokosawa et al., 1982, 1986; Azumi et al., 1987
		Japan Hokkaido		HC	Azumi et al., 1991
H. hispida	A	South Australlia	S	PL	Coombe et al., 1984
Herdmania momus	A	South Australlia	S	PL	Coombe et al., 1984
H. mirabilis	A	South Korea	S	HL	Present study
Microcosmus slucatus			S	PL	Bretting and Renwrantz, 1973
M. nichollsi	A	South Australlia	S	PL	Coombe et al., 1984
Pyura praeputialis	A	South Australlia	S	PL	Coombe et al., 1984
P. irregularis	A	South Australlia	S	PL	Coombe et al., 1984
P. sacciformis	A	South Korea	S	HL	Present study

A, Hemogglutinin. L, Hemolysin. N, none. S, Solitary. C, Colony. HL, Hemolymph. HC, Hemocyte. PL, Plasma. EP, Epithelium

Both humoral factors (hemoagglutinins and hemolysin) were present in most species, although the relative abundance varied from species to species, whereas no variation within the species existed among individuals. *Dendrodoa aggregata, Halocynthia roretzi, H. hilgendorfi igaboja, H. hilgendorfi ritteri, H. cactus, Chelyosoma siboga, Herdmania mirabilis,* and *Pyura sacciformis* agglutinated all three blood types and did not require divalent cations for agglutination, while lectins from *Styela plicata* and *Styela clava clava* agglutinated strongly B type than other A or O erythrocytes. They showed similar lytic activities. *Styela plicata, S. clava clava, Botryllus tuberatus,* and *Ciona intestinalis* contained both hemagglutinin and hemolysin. *Ciona intestinalis* contained humoral factors with strong lytic activity to human A, B, and O erythrocytes. In addition, there was cytotoxic activity shown rapidly within 1 min and there was weak hemagglutining activity against human A, B, and O erythrocytes. The ascidian, *Didemum* sp. inhibited powerful agglutining activity within 1 min. These results are presented in Table 1. It appeared that there was a variety of agglutining and lytic activities in the haemolymph of many different ascidians, although considerable variations in titer were observed.

Discussion

The occurrence of the humoral factors have been reported from 11 families of ascidians in the world (Table 2). There were no distributions in taxonomic patterns between the two orders or 11 families of world ascidians in solitary or colony form. No geographic variations were observed. Coombe et al. (1984) obtained hemolymph from 22 different ascidian species of South Australia and compared with agglutining activity against a panel of erythrocytes from sheep, mouse, guinea pig, trout, and tortoise. Humoral factors were not detected in four hemolymph samples. In *Ciona intestinalis*, the hemolymph had strong lytic but weak hemagglutining activities. Parrinello et al. (1993) reported that the hemocytes of *Ciona intestinalis* had natural cytotoxic capacity against mammalian erythrocytes (rabbit, guinea pig, and human A, B and O erythrocytes) *in vitro*. They suggested that these lytic factors were released from hemocytes and there was hemocyte specificity. Vasta et al. (1982) tested the distribution of plasma lectins in 10 species of North American ascidians. Yokosawa et al. (1986), Azumi et al. (1990), Suzuki et al. (1990) and Kawamura et al. (1991) isolated and characterized properties of humoral factors in Japanese ascidians. All except four species of ascidians in the world including Korea possessed humoral factors which suggest that the distribution of humoral factors in ascidians does not depend on geographical location. It means that the humoral factors play an important role in ascidian biological defense system.

Even in the same individual ascidian, there are many different kinds of humoral factors. They varied in their molecular weight, source (hemolymph, cellular), binding specificity and functions. The humoral and celllular agglutinins of the ascidian quite varied in molecular weight such as 14 kDa - 660 kDa (Wright and Cooper, 1984; Yokosawa et al., 1986; Azumi et al., 1990; Suzuki et al., 1990; Kelly et al., 1993), in carbohydrate-binding specificity toward galactose, or N-acetyl-D-galactosamine, and in a wide variety of functions which are agglutining, cytotoxicity, opsoning and budding. Recently, several agglutinins were isolated from the hemolymph and hemocytes, such as *Didemnum candium* lectin I and II, lectins from *Halocynthia roretzi*, *Styela clava* and *Phallusia mammilata*. They are different in molecular weights, isoelectric points and their hemagglutinating titers (Vasta et al., 1986a, b; Azumi et al., 1990; Kelly et al., 1993).

From *Botylloides leachii*, two kinds of lectins were isolated: the Ca^{++}-independent galactose-specific lectin with low molecular weight and the Ca^{++}-dependent galactose specific lectin of large molecules (Schluter et al., 1981). The galactose-specific Ca^{++}-depedent hemagglutinin was also isolated from the hemolymph of *Halocynthia roretzi* and *Ascidia malaca* (Yokosawa et al., 1986). Suzuki et al. (1990) had purified a galactose-specific lectin with 14 kDa from *Polyandrocarpa misakiensis*. Its amino acid sequence has 20-30% homology with those of mammalian lectins that associated with inflammation and lymphocyte homing receptor. The domain of ascidian hemagglutinins has cell adhesion property and it appears specifically during budding (Kawamura et al., 1991). Colonial ascidians propagate asexually by budding and strobilation, and they have an extensive capacity for regeneration by these humoral factors. In some colonical (*Botryllus*) ascidians the humoral factors show the colony specificity for fusion or nonfusion between colonies.

While the classification of ascidians falls into solitary and colonial ascidians, further studies are required to characterize the physiochemical properties and binding specifities of humoral factors, hemagglutinins and hemolysin. Humoral factors have been found in both solitary and colonial ascidians. Therefore, humoral factors of ascidian are not corresponded the orthodox taxonomic classification.

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References

Azumi K, Yokosawa H, and Ishii SI (1990) Halocyamines: novel antimicrobial tetrapeptide-like substances isolated from the hemocytes of the solitary ascidian *Halocynthia roretzi*. *Biochemistry* 29: 159-165.

Coombe DR, Ey PL, and Jenkin CR (1984) Ascidian haemagglutinins: incidence in various species, binding specificities and preliminary characterisation of selected agglutinins. *Comp Biochem Physiol* 77B: 811-819.

Fuke MT and Sugai T (1972) Studies on the naturallu occuring hemagglutinin in the coelomic fluid of an ascidian. *Biol Bull* 143:140-149.

Kawamura K, Fujiwara S, and Sugino YM (1991) Budding-specific lectin induced in epithelial cells is an extracellular matrix component for stem cell aggregation in tunicates. *Development* 113: 995-1005.

Kelly KL, Cooper EL, and Raftos A (1993) A humoral opsonin from the solitary urochordate *Styela clava*. *Dev Comp Immunol* 17: 29-39.

Kott P (1985) The Australian Ascidiacea. Part I. Phlebobrancia and Stolidobrancia. *Mem Queensl Mus* 23: 1-438.

Parrinello N (1996) Cytotoxic activity of tunicate hemocytes. In: Rinkevich B, and Müller WEG (eds), Invertebrate Immunology, Progress in Molecular and Subcellular Biology, Springer-Verlag, Berlin, pp 190-217.

Parrinello N, Arizza V, Cammarata M, and Parrinello DM (1993) Cytotoxic activity of *Ciona intestinalis* (Tunicata) hemocytes: properties of the *in vitro* reaction against erythrocyte targets. *Dev Comp Immunol* 17: 19-27.

Renwrantz L, and Uhlenbruck G (1974) Blood-group-like substances in some marine invertebrates. *Vox Sang* 26: 385-391.

Rho BJ (1975) On the classification and the distribution on the marine benthic animals in Korea. 3. Ascidians. *J Korean Res Inst Bet Liv* Ewha Womans Univ, Korea 15: 121-169.

Rho BJ (1995) The ascidians (Tunicata) from Chindo Islands, Korea. *Korean J Syst Zool* 11: 125-145.

Rho BJ, Choe BL, and Song JI (1996) Biosystematic studies on the marine fouling invertebrates in Korea: a systematic study on the ascidians from Chundo Island (Onsan Bay), Korea. *Korean J Syst Zool* 12: 221-235.

Rho BJ and Huh MK (1984) A systematic study on the ascidians in Korea. *J Korean Res Inst Bet Liv* Ewha Womans Univ, Korea 33: 99-136.

Rho BJ and Lee JE (1989) A systematic study on the ascidians from Cheju Island, Korea. *Korean J Syst Zool* 5: 59-76.

Rho BJ and Lee JE (1991) A systematic study on the ascidians in Korea. *Korean J Syst Zool* 7: 195-220.

Saito Y (1996) Humoral factors in tunicates. In: Rinkevich B and Muller WEG (eds), Invertebrate Immunology, Progress in Molecular and Subcellular Biology, Springer-Verlag, Berlin, pp 218-234.

Schluter SF, Ey PL, Keough DR, and Jenkin CR (1981) Identification of two carbohydrate-specific erythrocyte agglutinins in the haemolymph of the protochordate *Botrylloides leachii*. *Immunology* 42: 241-250.

Suzuki T, Takagi T, Furukohri T, Kawamura K, and Nakauchi M (1990) A calcium-dependent galactose-binding lectin from the tunicate *Polyandrocarpa misakiensis*. *J Biol Chem* 265: 1274-1281.

van Name WG (1945) The North and South American ascidians. *Bull Am Mus Nat Hist* 84: 1-476.

Vasta GR, Hunt JC, Marchalonis JJ, and Fish WW (1986a) Galactosyl-binding lectins from the Tunicate *Didemnum candidum*-Purification and physiochemical characterizaiton. *J Biol Chem* 261: 9174-9181.

Vasta GR and Marchalonis JJ (1986b) Galactosyl-binding lectins from the Tunicate *Didemnum candidum*: carbohydrate specificity and characterization of the combining site. *J Biol Chem* 261: 9182-9186.

Vasta GR, Warr GW, and Marchalonis JJ (1982) Tunicate lectins: distribution and specificity. *Comp Biochem Physiol* 73B: 887-900.

Wright RK and Cooper EL (1984) Protochordate immunity. II. Diverse hemolymph lectins in the solitary tunicate *Styela clava*. *Comp Biochem Physiol* 79B: 269-277.

Yokosawa H, Harada K, Igarashi K, Abe Y, Takaahashi K, and Ishi SI (1986) Galactose-specific lectin in the hemolymph of solitary ascidian, *Halocynthia roretzi*. Molecular, binding and functional properties. *Biochim Biophy Acta* 870: 242- 247.

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