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Agglutination of Bacteria by a Lectin in the Hemolymph of the Pacific Oyster *Crassostrea gigas*

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Summary

Cell-free hemolymph (plasma) from the Pacific oyster, *Crassostrea gigas*, contains two lectins with the ability to agglutinate ERBC (Gigalin E) and human RBC (Gigalin H). We investigated whether *C. gigas* plasma can agglutinate nine bacterial species, including five marine bacteria. All vertebrate erythrocytes (RBCs) tested (ERBC, GRBC and SRBC) were agglutinated by *C. gigas* plasma. Only Gigalin E required calcium ions for hemagglutinating activity (HA). *A. globiformis*, *B. subtilis*, *D. aquamarina*, *E. coli*, *S. putrefaciens* and *S. aureus* were agglutinated by *C. gigas* plasma, whereas *M. halophilus*, *V. alginolyticus* and *V. tubiashii* were not. Pre-incubation of *C. gigas* plasma with GRBC resulted in strongly reduced titers when the adsorbed plasma was titrated for agglutinating activity toward live cells of the three bacterial species. In contrast, pre-incubation with ERBC had no effect on subsequent agglutination of bacteria. The agglutinating activity of fixed cells of *S. aureus* did not change due to pre-adsorption with either ERBC or GRBC. These results indicate that the hemolymph component(s) responsible for erythrocyte agglutination also appear to be involved in bacterial agglutination. One component is probably Gigalin H, as indicated by the adsorption of hemagglutinating activity by GRBC.

Key words: lectin, hemolymph, agglutination, oyster, *Crassostrea gigas*

Lectins are protein complexes with carbohydrate-specific binding properties that are widely expressed in plants, invertebrates, and vertebrates and may serve a wide variety of physiological functions (Sharon and Lis, 1972). Invertebrates are believed to possess efficient host defense mechanisms by virtue of their humoral defense molecules because they lack antibody-mediated acquired immune systems (Arason, 1996). Lectins may act as recognition molecules for host defense activities such as aggregation and opsonization in invertebrates, including bivalve molluscs (Vasta and Marchalonis, 1984; Vasta *et al.*, 1994; Olafsen, 1995; Wang *et al.*, 2007). Lectins are good candidates for the recogni-

tion role because they can bind and opsonize foreign material with recognition specificity for 'pathogen-associated molecular patterns' (PAMPs) (Medzhitov and Janeway, 2000).

Invertebrate lectins have been found in hemolymph plasma and bound to hemocyte membranes (Vasta *et al.*, 1982 ; Renwrantz and Stahmer, 1983 ; Tasumi and Vasta, 2007). Lectins have been isolated and characterized from the hemolymph of many species of bivalve molluscs. In oysters, two lectins (Gigalins E and H) have been purified from the Pacific oyster, *Crassostrea gigas* (Hardy *et al.*, 1977 ; Olafsen *et al.*, 1992), and a calcium-dependent lectin (C-type lectin) has been purified from the Chilean oyster, *Ostrea chilensis* (Minamikawa *et al.*, 2004). In addition, lectin genes in the Pacific oyster have been analyzed. The full coding sequences of C-type lectin (CgCLec-1) and galectin (CgGal) from *C. gigas* have been obtained, and tissue expression analyses have been conducted (Yamaura *et al.*, 2008). Some bivalve lectins act as opsonins (Renwrantz and Stahmer, 1983 ; Olafsen *et al.*, 1992) and agglutinate environmental bacteria (Arimoto and Tripp, 1977 ; Tamplin and Fisher, 1989 ; Fisher and DiNuzzo, 1991 ; Tunkijjanukij *et al.*, 1997). Therefore, lectins appear to play a critical role in the host-defense mechanisms of bivalve molluscs, both by recognizing, binding to and agglutinating foreign microorganisms and by opsonizing foreign particles for uptake by phagocytic hemocytes.

A number of lectins described to date has been limited by the use of vertebrate erythrocytes (red blood cells ; RBCs) in agglutination assays (Fisher and DiNuzzo, 1991). RBCs are obviously not potential invaders. Furthermore, RBC surfaces exhibit only 7 of over 100 monosaccharides found in nature (Yeaton, 1981). In marine bivalve molluscs, investigation into the functional role of lectins must employ potent invasive organisms, such as marine bacteria. Gigalins E and H from the hemolymph of *C. gigas* agglutinate bacteria, including *Vibrio anguillarum* (Olafsen *et al.*, 1992) ; however, little is known about the interactions of *C. gigas* lectins with marine bacteria (Mori *et al.*, 1984). We investigated whether *C. gigas* plasma can agglutinate marine bacteria, including pathogenic *Vibrio* species.

Materials and Methods

Animals

Specimens of the Pacific oyster, *Crassostrea gigas*, with an average shell height of 12.4 ± 2.7 cm were obtained from hanging-culture beds in Matsushima and Onagawa Bays, Miyagi Prefecture, Japan. They were then transferred to our laboratory and held in 180-l aquariums filled with circulating, filtered artificial seawater (MARINE ART BR, Senju Seiyaku Co., Osaka, Japan) for five to seven days. Water temperature was maintained at 15 ± 1°C.

Preparation of Cell-free Hemolymph

Hemolymph was withdrawn from the adductor muscle using a tuberculin syringe with a 26-gauge, 0.5-inch needle. The collected hemolymph was centrifuged at $290 \times g$ for 10 min at 4°C to remove the hemocytes. After centrifugation, the supernatant (plasma) was used for the hemagglutination assays.

Erythrocytes

Hemagglutination assays of the plasma samples were performed using animal RBCs. Equine blood, goose blood and sheep blood stored in Alsever's solution were obtained from Nippon Biotest Laboratories (Tokyo, Japan).

Hemagglutinating Activity (HA) Assay

Equine erythrocytes (ERBC), goose erythrocytes (GRBC) and sheep erythrocytes (SRBC) used as agglutination test particles were placed in u-bottomed 96-well microtiter plates. Twelve oysters were used for this assay. A plasma sample (25 μl) was serially diluted two-fold with TBS buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl) or Ca-TBS buffer (TBS plus 10 mM $CaCl_2$, pH 8.0). The sample was then mixed with a 2% erythrocyte suspension (25 μl) in TBS. Activity was expressed as hemagglutinating (HA) titer, defined as the reciprocal of the highest dilution of plasma giving a positive reaction after 2 h at room temperature (21-24°C). Median HA titer was calculated and recorded. Each assay was performed in duplicate. Controls received TBS or Ca-TBS in place of oyster plasma.

Cross-adsorption Assays

Lectins in *C. gigas* plasma were examined for cross-reaction with ERBC, GRBC and SRBC. For each test, a 200-μl plasma sample was added to a tube containing 400 μl of packed RBCs. Tubes were gently rotated for 2 h at 4°C and then centrifuged at $500 \times g$ for 5 min at 20°C. The resulting supernatant was placed in microtiter plates, serially diluted, and tested by HA assays with fresh RBCs. Twenty oysters were used for this assay. Each assay was done in duplicate.

Bacteria and Culture Conditions

Nine selected bacterial strains were examined for agglutination by hemolymph plasma: *Arthrobacter globiformis* (IAM 12137), *Bacillus subtilis* (IAM 1026), *Deleya aquamarina* (IAM 12645), *Escherichia coli* (IAM 1264), *Marinococcus halophilus* (IAM 12844), *Shewanella putrefaciens* (IFO 3908), *Staphylococcus aureus* (IFO 3761), *Vibrio alginolyticus* (ATCC 19108) and *V. tubiashii* (ATCC 19106). Five of these species were marine bacteria: *D. aquamarina*, *M. halo-*

philus, S. putrefaciens, V. alginolyticus and V. tubiashii. IAM strains were kindly supplied by the Institute of Molecular and Cellular Biosciences of The University of Tokyo, Japan. IFO strains were purchased from the Institute for Fermentation, Osaka, Japan. ATCC strains were obtained from the American Type Culture Collection, Rockville, MD, USA. Before bacterial agglutinating activity (BA) assays, marine bacterial strains were cultured on marine agar 2216 (Difco Laboratories, Detroit, MI, USA) at 26°C. The four non-marine strains were cultured on tryptic soy agar (Difco Laboratories) at 26°C. After two days of growth, colonies were suspended in sterile balanced salt solution (BSS) formulated to be compatible with oyster physiology (446.6 mM NaCl, 14.5 mM KCl, 14.2 mM $MgSO_4 \cdot 7H_2O$, 10.6 mM $MgCl_2 \cdot 6H_2O$, 8.6 mM $CaCl_2$, 3.0 mM $NaHCO_3$, 0.08 mM NaH_2PO_4 and 5.6 mM glucose, pH 7.8; Takahashi and Mori, 2000) at approximately 10^9 colony-forming units (CFUs)/ml.

For some experiments, *S. putrefaciens* and *S. aureus* were treated with 1% glutaraldehyde for 12 hrs at room temperature. After fixation, the bacterial cells were washed three times and resuspended in BSS. This experiment demonstrated that glutaraldehyde fixation did not affect BA.

BA Assay

We determined the ability of cell-free oyster plasma to agglutinate bacteria. First, 50 μl of plasma was serially diluted two-fold with Ca-TBS in flat-bottomed 96-well plates, mixed with the bacterial suspension (25 μl, 10^9 CFU/ml) and incubated for 2 h at 25°C with gentle shaking every 30 min. After incubation, the resulting plates were observed under an inverted microscope at 400× magnification (Eclipse TE1000; Nikon Corp., Tokyo, Japan) to determine whether the bacterial cells were agglutinated. Five replicates of each agglutination assay were performed.

To determine the agglutinating specificity of lectins for bacteria, we also performed BA assays using supernatant pre-adsorbed with RBCs (ERBC and GRBC). Live cells of *A. globiformis, D. aquamarina* and *S. putrefaciens* and glutaraldehyde-fixed cells of *S. aureus* were used in these assays. Each assay was done in duplicate. Five oysters were used for these assays.

Results and Discussion

Hemagglutinating Specificity and Variability

Hemolymph from *C. gigas* contained lectins with agglutinating activity for a variety of cells, including a panel of RBCs. All RBCs tested (ERBC, GRBC and SRBC) were agglutinated by *C. gigas* plasma (Table 1). A survey of 12 individual oysters showed much higher HA titers for ERBC, ranging from 1,024 to 65,536 (median, 16,384; $n=12$), than for the other two RBCs. Interestingly, only

Table 1. Agglutination of three vertebrate erythrocytes (RBCs) by C. gigas plasma

	Agglutination with erythrocyte		
	Equine	Goose	Sheep
Plasma with Ca^{2+}	16,384 (512-65,536)	128 (16-512)	2,048 (0-8,192)
Plasma	2 (0-8)	128 (8-512)	1,024 (0-4,096)

Values in the Table demonstrate median hemagglutinating (HA) titers and range of HA titers of different samples ($n=12$).

ERBC agglutinating activity in *C. gigas* plasma required Ca^{2+}. Plasma from different individual oysters varied widely in HA titer for SRBC. Five of twelve individual oysters had very high titers, whereas the remaining oysters showed low or zero titers for SRBC. Previous studies have shown that *C. gigas* hemolymph contains two erythrocyte lectins with the ability to agglutinate ERBC (Gigalin E) and human RBC (Gigalin H) (Hardy *et al.*, 1977). Most C-type lectins identified in invertebrates are Ca^{2+}-dependent (Wang *et al.*, 2007). Thus, Gigalin E might be a C-type lectin.

Adsorption Studies

Plasma lectins demonstrated agglutinating activity for a variety of RBCs (Table 2). ERBC incompletely adsorbed HA activity for SRBC and did not adsorb that for GRBC. SRBC variably left a proportion of HA activity unadsorbed. GRBC adsorbed most HA activity for SRBC, but HA activity for ERBC was unaffected. It has been observed that GRBC completely adsorbs HA activity for HRBC (Nakamura, unpublished data). Therefore, HA activity for GRBC was produced by Gigalin H. This study reconfirms that two lectins, Gigalins E and H, occur in *C. gigas* hemolymph.

Agglutination of Bacteria

To establish whether *C. gigas* plasma lectins play a role in preventing

Table 2. Cross-adsorption tests of C. gigas plasma with different RBCs

Adsorbing RBC	HA titer		
	ERBC	GRBC	SRBC
ERBC	-	128	64
GRBC	16,384	-	256
SRBC	8	64	-
None (Ca-TBS only)	16,384	128	512

Values in the Table demonstrate median HA titer ($n=20$). -; HA titers were not detected.

Table 3. Bacterial agglutinating (BA) activity of C. gigas plasma toward nine bacterial strains

Treatment	Strain	Median BA titer	Range
Live	*Arthrobacter globiformis*	1,024	512–2,048
	Bacillus subtilis	8	4–16
	Deleya aquamarina	32	16–128
	Escherichia coli	4	4–8
	Marinococcus halophilus	-	-
	Shewanella putrefaciens	512	512–1,024
	Staphylococcus aureus	4	2–4
	Vibrio alginolyticus	-	-
	Vibrio tubiashii	-	-
Fixed	*Shewanella putrefaciens*	-	-
	Staphylococcus aureus	64	64–128

-; not detected.

bacterial infection, we investigated the ability of *C. gigas* plasma to agglutinate bacteria, including pathogenic species. *A. globiformis*, *B. subtilis*, *D. aquamarina*, *E. coli*, *S. putrefaciens* and *S. aureus* were agglutinated by *C. gigas* plasma, whereas *M. halophilus*, *V. alginolyticus* and *V. tubiashii* were not (Table 3). Agglutinating activities for *A. globiformis* and *S. putrefaciens* were remarkably high. *S. putrefaciens* cells were not agglutinated by fixation with glutaraldehyde, while fixed cells of *S. aureus* were strongly agglutinated. Because the agglutinating abilities of lectins are heterogeneous in structure and ligand binding, different bacterial surface moieties may be involved in this process (Canesi *et al.*, 2002). Gigalins E and H from *C. gigas* hemolymph agglutinate bacteria, including *Vibrio anguillarum* (Olafsen *et al.*, 1992). In the American oyster (*C. virginica*), Tamplin and Fisher (1989) have reported that *V. cholerae* strains belonging to O1 serovars and biovars are predominantly agglutinated by cell-free plasma, but 79 other bacterial strains, representing 26 species, are not agglutinated. Furthermore, these authors also indicate that some other *Vibrio* species, such as *V. vulnificus* and *V. parahaemolyticus*, are not agglutinated by *C. virginica* plasma. Similar results have been obtained by Fisher and DiNuzzo (1991). In this study, bacteria that are pathogenic for bivalve larvae, *V. alginolyticus* and *V. tubiashii*, were not agglutinated by *C. gigas* plasma. The effects of oyster lectins on the ecology of vibrios in marine and estuarine waters remain unclear.

Pre-incubation of *C. gigas* plasma with GRBC resulted in strongly reduced titers when the adsorbed plasma was titrated for agglutinating activity toward live cells of the three bacterial species (Table 4). In contrast, pre-incubation with ERBC had no effect on subsequent agglutination of bacteria. The agglutinating

Table 4. BA titer of C. gigas plasma following adsorption with ERBC and GRBC

Treatment	Strain	RBC	Median BA titer	Range
Live	*Arthrobacter globiformis*	ERBC	1,024	512-2,048
		GRBC	4	0-16
		None	1,024	512-2,048
	Deleya aquamarina	ERBC	64	32-64
		GRBC	2	0-2
		None	64	32-128
	Shewanella putrefaciens	ERBC	256	256-512
		GRBC	4	2-16
		None	256	256-512
Fixed	*Staphylococcus aureus*	ERBC	64	32-128
		GRBC	32	16-128
		None	64	64-128

activity of fixed cells of *S. aureus* did not change due to pre-adsorption with either ERBC or GRBC. These results indicate that the hemolymph component(s) responsible for erythrocyte agglutination also appear to be involved in bacterial agglutination (BA). One component is probably Gigalin H, as indicated by the adsorption of hemagglutinating activity by GRBC. Gigalin H was almost completely removed with GRBC by centrifugation, and BA activity of the resulting supernatant was markedly reduced for live cells of all bacterial species tested. Gigalin H exhibits specificity for sialic acid high affinity for sialic acid residues in glycoproteins (Hardy *et al.*, 1977). In a study of *C. virginica* lectins, the *V. cholerae* ligand has been suggested to be a heat-stable N-acetyl-neuramic acid-like residue (Tamplin and Fisher, 1989). Cross-adsorption tests showed that *C. gigas* plasma incubated with ERBC eliminated only HA, and BA titer was not reduced. Gigalin E apparently did not agglutinate the bacteria examined in this study. BA ability for fixed cells of *S. aureus* was not reduced by pre-treatment with RBCs. This result implies that HA and a part of BA activity may involve different components of *C. gigas* hemolymph. As described above, previous investigations have found only two lectins (Gigalins E and H) in *C. gigas* hemolymph (Hardy *et al.*, 1977; Olafsen *et al.*, 1992). It remains to be determined whether other agglutinating substances are present in *C. gigas* hemolymph.

References

Arason, G.J., *Fish Shellfish Immunol.*, **6**, 277-289 (1996)
Arimoto, R. and Tripp, M.R., *J. Invertebr. Pathol.*, **30**, 406-413 (1977)

Canesi, L., Gallo, G., Gavioli, M., and Pruzzo, C., *Micros. Res. Tech.*, **57**, 469-476 (2002)
Fisher, W.S. and DiNuzzo, A.R., *J. Invertebr. Pathol.*, **57**, 380-394 (1991)
Hardy, S.W., Grant, P.T., and Fletcher, T., *Experientia*, **33**, 767-769 (1977)
Medzhitov, R. and Janeway Jr., C., *Immunol. Rev.*, **173**, 89-97 (2000)
Minamikawa, M., Hine, M., Russell, S., Huber, P., Duignan, P., and Lumsden, J.S., Fish *Shellfish Immunol.*, **17**, 463-476 (2004)
Mori, K., Murayama, K., Kanno, N., Ñakamura, M., Ohira, E., Kato, Y., and Numura, T., *Tohoku J. Agr. Res.*, **35**, 55-68 (1984)
Olafsen, J.A., Fletcher, T.C., and Grant, P.T., *Dev. Comp. Immunol.*, **16**, 123-138 (1992)
Renwrantz, l. and Stahmer, A., *J. Comp. Physiol.*, **149**, 535-546 (1983)
Sharon, N. and Lis, H., *Science*, **177**, 949-958 (1972)
Takahashi, K.G. and Mori, K., *Tohoku J. Agr. Res.*, **51**, 5-27 (2000)
Tamplin, M.L. and Fisher, W.S., *Appl. Environ. Microbiol.*, **55**, 2882-2887 (1989)
Tasumi, S. and Vasta, G.R., *J. Immunol.*, **179**, 3086-3098 (2007)
Tunkijjanukij, S., Mikkelsen, H.V., and Olafsen, J.A., *Comp. Biochem. Physiol.*, **117B**, 273-286 (1997)
Vasta, G.R., Sullivan, J.T., Cheng, T.C., Marchalonis, J.J., and Warr, G.W., *J. Invertebr. Pathol.*, **40**, 367-377 (1982)
Vasta, G.R. and Marchalonis, J.J., "Recognition proteins, Receptors, and probes" ed. by G.R. Vasta and J.J. Marchalonis, A.R. Liss, New York, pp. 177-191 (1984)
Vasta, G.R., Cheng, T.C., and Marchalonis, J.J., *Cell. Immunol.*, **88**, 475-488 (1984)
Wang, H., Song, L., Li, C., Zhao, J., Zhang, H., Ni, D., and Xu, W., *Mol. Immunol.*, **44**, 722-731 (2007)
Yamaura, K., Takahashi, K.G., and Suzuki, T., *Comp. Biochem. Physiol.*, **149B**, 168-175 (2008)
Yeaton, R.W., *Dev. Comp. Immunol.*, **5**, 391-402 (1981)