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Partial Inhibition of Hemocyte Agglutination by *Lathyrus odoratus* Lectin in *Crassostrea virginica* Infected with *Perkinsus marinus*

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Quantitative determinations of agglutination of hemocytes from oysters, Crassostrea virginica, *by the* Lathyrus odoratus *lectin at five concentrations revealed that clumping of hemocytes from oysters infected with* Perkinsus marinus *is partially inhibited. Although the nature of the hemocyte surface saccharide, which is not D(+)-glucose, D(+)mannose, or α-methyl-D-mannoside, remains to be determined, it may be concluded that this molecule also occurs on the surface of* P. marinus.

It has been demonstrated that the panning technique (Ford et al. 1990) is qualitatively as effective for determining the presence of P. marinus *in* C. virginica *as the hemolymph assay method (Gauthier & Fisher 1990).*

Key words: *Crassostrea virginica* - oyster - *Perkinsus marinus* - *Lathyrus odoratus* - lectin - hemocytes

In an earlier study (Cheng et al. 1993), it was reported that there is a saccharide on the surface of hemocytes of the American oyster, *Crassostrea virginica*, from Apalachicola Bay, Florida, and Galveston Bay, Texas, USA, that binds to the *Lathyrus odoratus* (sweat pea) lectin. This sugar is neither D(+)-mannose nor D(+)-glucose, which are known inhibition sugars for *L. odoratus* lectin (Tichá et al. 1980). Subsequently, Cheng et al. (1994) reported that this unidentified sugar on hemocyte surfaces could serve as a marker for innate resistance in oysters to the pathogenic protistan parasite *Haplosporidium nelsoni* as it occurs in all hosts from Apalachicola Bay, Florida, where *H. nelsoni* has never been found, and in 78% of oysters from coastal South Carolina where, with rare exceptions, *H. nelsoni* does not occur in the same bivalves that include this saccharide on their hemocyte surfaces.

During studies parallel to those cited above, it was noticed that there appeared to be quantitative differences in the binding of the *L. odoratus* lectin to hemocytes of oysters infected and uninfected with another protozoan pathogen, *Perkinsus marinus*. The study being reported herein was subsequently carried out to confirm or negate this preliminary observation.

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MATERIALS AND METHODS

Oysters - All of the oysters, *C. virginica*, employed in this study were from Apalachicola Bay, Florida, USA. All were collected between June 15 and August 15, 1992. This time period was selected because it is known that there are relatively high prevalence and intensity per host of *P. marinus* in Florida oysters during this season (WS Fisher, pers. comm.). All oysters were held in the laboratory at 3°C in 15‰ artificial sea water until 1 hr prior to bleeding at which time they were removed from water and held at room temperature (24°C). None was held at 3°C for more than three days.

Hemolymph collection - Approximately 3 ml of whole hemolymph were collected from the adductor muscle sinus of each of 124 oysters by use of sterile 21 gauge hypodermic needles and 1 ml turberculin syringes. One ml of each sample was employed for the determination of the presence of *P. marinus* by use of the hemolymph assay method (Gauthier & Fisher 1990). The remaining 2 ml were employed in lectin studies. These were washed three times in isotonic (540 mOsm) saline (IS) involving centrifugation at 300 g in a table top centrifuge. After the third wash, the cell pellets were gently resuspended in 2 ml of IS. The final cell counts averaged 2-3 x 10^4/ml.

Lectins - The most concentrated solution of the *L. odoratus* lectin employed was 0.1 mg/ml. The purified lectin, as well as D(+)-glucose, D(+)-mannose, and α-methyl-D-mannoside, the known inhibitor saccharides for this lectin (Tichá et al. 1980), were purchased from Sigma (St. Louis, Missouri, USA). The lectin solutions were

prepared in phosphate-buffered saline and were serially diluted 2-fold with IS in microtiter plates to give final dilutions of 1:1 to 1:2048. The agglutination tests were carried out in 96 well U-bottom plates (Cell wells, Corning, New York, USA). To test possible inhibition by D(+)-glucose, D(+) mannose, and α-methyl-D-mannoside, the most concentrated lectin solution was serially diluted in 0.2 M solutions of the three saccharides.

In addition to the *L. odoratus* lectin, concanavalin A, type III (Con A) was included in every test as a positive control as it is known that it will agglutinate *C. virginica* hemocytes (Yoshino et al. 1979, Cheng et al. 1980, 1993, 1994, Kanaley & Ford 1990). Con A was also purchased from Sigma, as was N-acetyl-D-glucosamine, the inhibiting saccharide employed. The most concentrated solution of Con A tested was 1.0 mg/ml.

All of the hemocyte samples tested were from single oysters. A total of 114 samples were tested against both lectins. The cells from the remaining ten oysters were employed in negative control tests, i.e., IS, instead of lectin, was used. None of these resulted in agglutination of hemocytes.

Fifty µl of hemocyte suspension were added to each experimental and control well and the plates were incubated for 24 hr at room temperature (24°C).

As earlier studies (Cheng et al. 1980, 1993, 1994) have revealed that not all of the hemocytes exposed to selected lectins, including the *L. odoratus* lectin, agglutinated, we ascertained the percentages of clumped and single cells at the highest concentration of *L. odoratus* lectin tested as well as at four dilutions: 1:64, 1:512, 1:1024, and 1:2048. The counting of agglutinated and single cells was achieved on three samples at each dilution with phase-contrast microscopy. When three or more cells were clumped, these were considered to be agglutinated. Pairs were seldom observed.

Detection of P. marinus - To determine the possible occurrence of *P. marinus*, as stated, the hemolymph assay method of Gauthier and Fisher (1990) was employed. Briefly, whole hemolymph samples were individually centrifuged at 265 g for 5 min after which the serum was decanted. The pellets (containing hemocytes and *P. marinus* life cycle stages, if present) were resuspended in 1 ml of fluid thioglycolate medium with 5 ml of Mycostatin and 5 ml of Chloromycetin (Ray 1966). The cultures were maintained in the dark at 26 °C for five days after which the culture medium was removed by centrifugation. The pellets were each resuspended in 1 ml of 2M NaOH, which reduced interference caused by bacteria and hemocytes without disrupting *P. marinus* hypnospores (Choi et al. 1989). After washing with distilled water, the samples were stained with Lugol's iodine solution and the presence or absence of stained life cycle stages was determined microscopically. Also, during the examination of samples from the agglutination plates, confirmation of the presence or absence of *P. marinus* was carried out.

In addition to employing the hemolymph assay method of Gauthier and Fisher (1990), five additional oysters from Apalachicola Bay were similarly bled and 1 ml of whole hemolymph from each was subjected to hemolymph assay and an additional 1 ml of whole hemolymph was subjected to the panning technique of Ford et al. (1990), which was originally devised to detect the presence of *Haplosporidium nelsoni*, another pathogenic parasite of *C. virginica*. Briefly, this method takes advantage of the greater adherence of hemocytes, compared to protozoan parasites, to the bottom of Petri dishes. Hence, oyster hemolymph was layered in dishes and allowed to settle for 30 min at 26°C. Subsequently, non-adhering cells were examined microscopically for the identification of *P. marinus*.

RESULTS

Agglutination tests - The mean percentages and ranges of clumped and single hemocytes from uninfected oysters exposed to the five dilutions of *L. odoratus* lectin are presented in Table. Similar data pertaining to hemocytes from oysters infected with *P. marinus* exposed to the five dilutions of the lectin also are presented in Table.

Also presented in Table are the observations that the three saccharides, D(+)glucose, D(+)-mannose, and α-methyl-D-mannoside, do not inhibit the agglutination of hemocytes from uninfected oysters and those infected with *P. marinus* that had been exposed to the *L. odoratus* lectin. Also, the clumping of hemocytes by this lectin is diminished in both *P. marinus*-infected and uninfected oysters as the dilution of the lectin is increased (Table).

As indicated by our data pertaining to the clumping of hemocytes from uninfected oysters and those harboring *P. marinus*, there is no difference in the ability of Con A to agglutinate both categories of hemocytes. Also, the percentages of agglutinated cells decrease and those of single cells increase as the concentration of Con A is decreased (Table). Furthermore, the clumping of hemocytes at each of the five concentrations of Con A is inhibited by N-acetyl-D-glucosamine (Table).

Detection of P. marinus - Among the 114 oysters employed for lectin studies in which the presence or absence of *P. marinus* was determined by the hemolymph assay method of Gauthier and Fisher (1990), 88 (77%) were found to be infected. Among the additional five oyster hemolymph samples that were subjected to both

TABLE

Means and ranges of percentages of (clumped/single) hemocytes of *Perkinsus marinus*-infected and noninfected *Crassostrea virginica* from Apalachicola Bay, Florida, USA, treated with the *Lathyrus odoratus* lectin and Con A at five concentrations. The highest concentration (conc.) of *L. odoratus* lectin was 0.1 mg/ml and that of Con A was 1.0 mg/ml. inh, inhibition by saccharide indicated; ninh, not inhibited by saccharide indicated

			Lectin concentration				
Oysters	Lectin	Inhibition saccharide	conc.	1:64	1:512	1:1024	1:2048
Uninfected	*L. odoratus*		62	26	24	21	11
(n=26)			(29-87)	(12-58)	(0-68)	(0-53)	(0-26)
			38	74	76	79	89
			(13-84)	(42-94)	(32-100)	(47-100)	(74-100)
		D(+)-glucose	ninh	ninh	ninh	ninh	ninh
		D(+)-mannose	ninh	ninh	ninh	ninh	ninh
		α-methyl-D-mannoside	ninh	ninh	ninh	ninh	ninh
	Con A		95	65	45	38	6
			(82-100)	(48-72)	(36-65)	(19-48)	(0-12)
			5	35	55	62	94
			(3-10)	(24-43)	(43-62)	(40-66)	(79-100)
		N-acetyl-D-glucosamine	inh	inh	inh	inh	inh
Infected	*L. odoratus*		18	5	3	1	1
(n=88)			(0-53)	(0-40)	(0-26)	(0-12)	(0-7)
			82	95	97	99	99
			(39-100)	(60-100)	(74-100)	(88-100)	(93-100)
		D(+)-glucose	ninh	ninh	ninh	ninh	ninh
		D(+)-mannose	ninh	ninh	ninh	ninh	ninh
		α-methyl-D-mannoside	ninh	ninh	ninh	ninh	ninh
	Con A		93	58	44	32	7
			(82-100)	(48-72)	(36-65)	(19-48)	(0-12)
			7	42	56	68	93
			(3-8)	(20-58)	(40-68)	(42-86)	(80-100)
		N-acetyl-D-glucosamine	inh	inh	inh	inh	inh

the panning (Ford et al. 1990) and the hemolymph assay methods (Gauthier & Fisher 1990) for determining the possible presence of *P. marinus*, all were found to be parasitized by this protozoan. Thus, among a total of 119 oysters examined from Apalachicola Bay, Florida, during this study, 93 (78%) were infected with *P. marinus*.

DISCUSSION

The data presented in the Table indicate that there are decreases in the percentages of agglutinated hemocytes and increases in the percentages of single cells as the concentrations of the *L. odoratus* lectin decrease. This applies to the hemocytes of both uninfected oysters as well as those parasitized by *P. marinus*.

Also, it has been reaffirmed that D(+)-glucose, D(+)-mannose, and α-methyl-D-mannoside do not inhibit agglutination of oyster hemocytes from infected as well as uninfected oysters. This indicates that the saccharide on the surface of hemocytes of both categories of oysters is not one of these molecules. Its nature remains undetermined.

The reduction in the percentage of clumped hemocytes and increase in that of single cells after exposure to each of the five concentrations of *L. odoratus* lectin in the case of oysters infected with *P. marinus* (Table) indicate that the parasite is acting as an inhibitor. As lectins are inhibited by specific sugar residues, it is concluded that the yet to be identified saccharide to which *L. odoratus* lectin is bound on the surface of oyster hemocytes also occurs on the surface of *P. marinus*. Based on the concept of molecular mimicry (Damian 1964, 1979), this, and most probably other molecular similarities, may account for the fact that many of the *P. marinus* are recognized as self by the oyster host and consequently are not phayocytosed by its hemocytes.

In view of the findings being reported herein, it is predicted that in areas where *H. nelsoni* and *P. marinus* coexist, one would not expect to find the high percentages of agglutinated hemocytes when exposed to the *L. odoratus* lectin as reported earlier in the case of hemocytes from *H. nelsoni*-resistant oysters not infected with *P. marinus* (Cheng et al. 1994).

Finally, our results pertaining to the use of both the panning method (Ford et al. 1990) and the hemolymph assay method (Gauthier & Fisher 1990) indicate that both methods are equally as effective for qualitatively determining infection of *C. virginica* with *P. marinus*.

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