

**Studies on the Effects of Different Molluscan Shells on the Induction of
Larval Settlement of Pacific Oyster *Crassostrea gigas* and the
Characterization of a Larval Settlement Inducing Compound from
Conspecifics**

マガキ (*Crassostrea gigas*) 幼生の付着に対する各種貝殻の誘起効果
および同種貝殻由来の付着誘起物質に関する研究

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English Abstract

The Pacific oyster *Crassostrea gigas* is an economically important bivalve. It is native to Japan and Korea but it has been introduced to several countries worldwide mainly for aquaculture purposes. With an estimated production of 608,688 t in 2012, *C. gigas* has become the leading species in world oyster culture. In the production of *C. gigas*, much of the global supply of spat is obtained from wild seed capture. However, when natural seed is not available, spats are obtained from hatchery cultured larvae. Hence researchers have been interested in the elucidation of the settlement inducer of *C. gigas* larvae. In this study, settlement of *C. gigas* larvae on shells of different species of mollusks was investigated. Moreover, the settlement-inducing compound in the shell of *C. gigas* was characterized and partially purified.

In Chapter II, the settlement inducing activity of 11 species of mollusks on larvae of *C. gigas* were investigated. *C. gigas* larvae settled on SC of all species tested except on *Patinopecten yessoensis* and *Atrina pinnata*. In SC of species that induced *C. gigas* larvae to settle, settlement was proportionate to the amount of SC supplied to the larvae. When compared to *C. gigas* SC, all species except *C. nippona* showed lower settlement inducing activities, suggesting that the cue may be more abundant or in a more available form to the larvae in shells of conspecific and *C. nippona* than in other species. Settlement-inducing activity of *C. gigas* SC remained stable up to 200°C but was significantly reduced at 300°C. In addition, antibiotic treatment did not affect the activity of the SC.

C. gigas SC extracts prepared with water (Aq-ex), ethanol (EtOH-ex) and diethyl ether (Et₂O) did not induce larval settlement of *C. gigas*

larvae. However, SC extract using hydrochloric acid (HCl-ex) induced high larval settlement that was at the same level as the SC. Dialyzing and freeze-drying the acid extract (FD HCl-ex) did not change its settlement inducing activity.

Settlement of *C. gigas* larvae on nitrocellulose membrane, plaster plate, GF/C filter paper and glass were investigated. All substrates as-is did not induce larval settlement but when FD HCl-ex was added to each of these substrates, larvae settled on all substrates but highest settlements were observed on the GF/C filter papers.

In Chapter III, the FD HCl-ex was subjected to heat, enzymatic (pepsin, trypsin, PNGase F) and chemical (trifluoromethanesulfonic acid [TFMS], lectins) treatments and the effects of these treatments on the inducing activity of FD HCl-ex were investigated. The settlement inducing activity of FD HCl-ex was stable at 100°C but was destroyed or degraded after pepsin, trypsin, PNGase F and TFMS treatments indicating that the settlement cue was a glycoprotein. *C. gigas* larval settlement on SC and FD HCl-ex in the presence of wheat germ agglutinin (WGA), soybean lectin (SBA), lectin lectin (LCA) and concanavalin A (Con A) was investigated and was found to decrease in a concentration dependent manner only in the presence of WGA. However, the inhibitory effect of WGA on the activity of the FD HCl-ex was canceled by N-acetyl-D-glucosamine. On the other hand, SC and FD HCl-ex dyed with fluorescein isothiocyanate conjugated WGA exhibited fluorescence under the UV view. Fractionation of the FD HCl-ex in Superdex 200 10/300 GL eluted an active fraction between the molecular mass range of 45 and 150kDa and revealed a major band at 55kDa on the SDS-PAGE.

From the above results, the author concluded that *C. gigas* larvae settled on shells of different species of mollusks but settled in higher numbers on shells of its conspecifics and *C. nippona*. The settlement cue in conspecific shells is a heat stable 55 kDa glycoprotein that is insoluble in water. Moreover, the settlement cue has a WGA-binding sugar chain that plays an important role during settlement of *C. gigas* larvae. This study is the first to provide evidence on the chemical basis of *C. gigas* larval settlement on conspecific shells.

要旨

マガキはカキ目、イタボガキ科に属し、沿岸域に生息する二枚貝である。本種は、日本、韓国が原産国であるが、養殖を目的に多くの国に導入され、現在は世界中で盛んに養殖されている。日本は世界有数のマガキ生産国であるが、養殖は主に天然種苗を用いて行われている。海外では、人工種苗生産による養殖が主流のため、付着誘起物質の探索および幼生の付着メカニズムの解明に関心がある。本種の幼生はバクテリア、同種個体によって付着が誘起されることがすでに報告されており、同種個体由来のケミカルシグナルが群居性の化学的根拠になっていると考えられている。また、海外の養殖施設では本種の群居性を利用した採苗方法として幼生の付着基質に同種貝殻が用いられている。しかし、同種個体由来の付着誘起物質は未だ不明である。

本研究では、本種の群居性に着目して同種個体由来の付着誘起物質を特定するために、まず、本種幼生に対して各種貝殻の付着誘起活性を比較するとともに本種貝殻に含まれる付着誘起物質を抽出した（第2章）。さらに、本種貝殻由来の幼生付着誘起物質について、化学的性質を検討するとともに部分精製を試みた（第3章）。

第2章では、11種類の貝殻よりシェルチップ（SC）を調製し、それぞれの本種幼生に対する付着誘起活性を調べた。その結果、幼生は同種およびイワガキのSCに対して高い付着率を示した。ホタテガイおよびタイラギのSCを除いてその他のSC全てが幼生の付着を誘起したが、SCの種類によって付着誘起活性が異なり、SCの

量も活性に関係していた。本種 SC は、活性が 200°C の熱処理では安定していたが、300°C では失活した。また、SC は抗生物質処理しても、活性が変わらなかった。本種 SC を種々の溶媒で抽出し、抽出液の活性を調べた結果、水、エタノール (EtOH) およびジエチルエーテル (Et₂O) の抽出液は活性が認められなかった。一方、HCl 抽出液 (HCl-ex) は SC に匹敵する高い活性を示し、透析・凍結乾燥した塩酸抽出液 (FD HCl-ex) も高い活性を示した。ガラス、ニトロセルローズ膜、石膏プレートおよびガラス繊維ろ紙 (GF/C) はいずれも幼生に対して付着誘起活性がなかったが、FD HCl-ex を塗布した結果、すべての基盤に対して幼生が付着し、GF/C では高い付着率がみられた。

第 3 章では、FD HCl-ex に対して温度、酵素 (ペプシン、トリプシン、N-グリコシダーゼ F)、薬剤 (トリフルオロメタンスルホン酸 (TFMS)、レクチン) の種々の処理を施し、FD HCl-ex の性質を調べた。温度処理実験では、FD HCl-ex は 100°C でも活性が変わらなかった。また、FD HCl-ex はペプシン、トリプシン、N-グリコシダーゼ F および TFMS の各処理によって活性が半減または消失し、活性本体は糖タンパク質であることが示唆された。さらに、FD HCl-ex をレンズ豆 (LCA)、コンカナバリン (ConA)、大豆 (SBA) および小麦胚芽 (WGA) の各種レクチンで処理した結果、WGA のみが濃度依存的に阻害した。しかし、その阻害効果は N-アセチルグルコサミンによって取り消された。FITC 標識 WGA を用いた蛍光法では、幼生の付着誘起に関与する糖タンパク質が SC および FD HCl-ex 中に含まれていることが観察できた。一方、FD HCl-

ex を Superdex 200 10/300 GL でゲル濾過した結果、3つの画分を回収し、そのうちの 45~150kDa 画分が幼生の付着を誘起した。活性を示した画分を SDS-PAGE で分析したところ、55kDa の位置に量の豊富なバンドがみいだされた。

以上の第2章および第3章の結果より、本種幼生は種々貝殻に付着するが、同種および近縁種のイワガキの貝殻が最も高い付着誘起活性を示した。本種の貝殻に含まれる付着誘起物質は、分子量 55kDa の糖タンパク質であり、水に不溶で熱に安定な物質であった。また、本付着誘起物質は WGA と結合する糖鎖部位をもち、同糖鎖部位が付着に関与していることが分かった。本研究は、本種幼生の付着が同種貝殻に誘起される（群居性）化学的根拠を初めて示した研究である。

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I. General Introduction

The Pacific oyster, *Crassostrea gigas* is an economically important species native to Japan and Korea. It has been introduced to a number of countries, including the US, Canada, United Kingdom, France, New Zealand, Australia, South Africa, and South America, mainly for aquaculture purposes (Orensanz et al. 2002; Ruesink et al. 2005). *C. gigas* has become the leading species in world oyster culture, with an estimated production of 608,688 t in 2012 (FAO/FIGIS). In the production of *C. gigas*, much of the global supply of spat is obtained from wild seed capture (FAO 2005-2014). However, when natural seed is not available, larvae are cultured and induced to metamorphose in hatcheries and the material used consists principally of *C. gigas* oyster shells (Breese and Malouf 1975; Jones and Jones 1988; Baltazar et al. 1999; Robert and Gerard 1999).

Pacific oysters are oviparous; gonadal development occurs at water temperatures above 23°C and spawning occurs at temperatures above 27-28°C (Kobayashi et al. 1997). During spawning, the eggs are discharged from the ovary into the mantle cavity, then, the abductor muscle undergoes a series of rhythmic contractions, causing the discharge of the egg through the opening of the mantle into the sea. The spermatozoa are released from between the partially opened valves of the shell and fertilization occurs in the sea (Fujiya 1970). In the Northern Hemisphere they release their gametes mainly in July and August, when water temperatures are highest (Troost 2010). After a pelagic phase of about 3 weeks, the pediveliger settle onto oyster shells, rocks, or pieces of other hard substrate and by secretion of cement the left cupped valve becomes attached to the substrate (Gosling 2003; Troost 2010).

There is substantial evidence that larvae of different oyster species (Pacific oyster, *C. gigas* [Weiner et al., 1989; Bonar et al., 1990; Coon et al., 1990a; Tamburri et al., 1992; Tritar et al., 1992]; American oyster, *C. virginica* [Weiner et al., 1989]; flat oyster *Ostrea edulis* [Tritar et al. 1992]) settle in response to biofilms. A two cue model of microbial biofilm induction of oyster settlement has been proposed by Weiner et al. (1989) where in ammonia released by bacteria in biofilm initially cues swim/search behavior (Coon et al., 1990b). While ammonia may induce larvae to sample the substrate, ammonia alone is not sufficient to entice the larvae to remain there. Another factor(s) in the biofilm cues crawl/search behavior and cementation, completing settlement. It is hypothesized that bacteria may signal the presence of a substratum that has been submerged in the sea for a long time enough to accumulate a substantial amount of biofilms and that the surface is generally non-toxic (Johnson et al. 1997). It might also be that the settling larvae actually employ the adhesive properties of bacteria to increase their own attachment strength (Hadfield 2011). However, the significance of biofilms on oyster larval settlement remains to be investigated.

Another source of settlement cue for oysters is conspecifics. Induction of larval settlement by conspecifics was first reported by Cole and Knight-Jones (1939) for *Ostrea edulis*. Bayne (1969) later confirmed the observation of Cole and Knight-Jones (1939) and reported that extracts of *O. edulis* tissue was effective in promoting larval settlement when this was applied to a surface. Since then, the inducing effect of conspecifics on larval settlement has been studied for other oyster species both in the laboratory and the field i.e., *Crassostrea virginica* (Crisp 1967; Hidu 1969; Veitch and Hidu 1971; Keck et al. 1971; Tamburri et al. 1996), *C. gigas*

(Hirata 1998; Hirata 2005; Tamburri et al. 2007), and *C. ariakensis* (Tamburri et al. 2008). In *C. virginica*, adult shells, water pre-conditioned by adults and “oyster shell liquor” have been shown to promote larval settlement (Crisp 1967; Keck et al. 1971; Tamburri et al. 1992). Veitch and Hidu (1971) further reported that the inducing substance present in the “oyster shell liquor” was a thyroxine containing protein with MW greater than 100,000 Da. Crisp (1967) observed that larvae of *C. virginica* settled almost entirely on conspecific shells but destruction of the organic layers on its surface clearly rendered the substratum unfavorable. The pre-treatment of scallop shell spat collectors with seawater containing live adult oysters increased the ratio of *C. gigas* larval settlement on spat collectors (Hirata 1998). *C. gigas* larvae have also been observed to settle preferentially on shells of conspecifics than shells of mussels, and larval settlement on shells of both living and dead conspecifics were the same (Diederich 2005).

Despite the interest on the elucidation of larval settlement cues of *C. gigas*, larval settlement cues remain poorly understood. In this study, the author investigated the effects of different molluscan shells on the induction of larval settlement of the Pacific oyster *Crassostrea gigas*. Moreover, the author partially characterized the larval settlement-inducing compound from conspecifics using a bioassay-guided approach.

In chapter II, the author investigated the settlement-inducing activities of shell of different molluscan species. The purpose was to check if different shells have different inducing activities on the *C. gigas* larval settlement. Simultaneously, settlement-inducing activities of extracts prepared from the conspecific oyster shells using different chemicals were

tested and the oyster shell extract was further applied on different substrates, as-is and when the oyster shell extract was added to them, in order to select a suitable substrate for settlement assays.

In chapter III, the oyster shell extract was subjected to physical and chemical treatments in order to characterize the settlement cue. Characteristics such as heat stability after extraction, proteinaceous nature, settlement in the presence of sugars and the role of lectins in the settlement triggered by shells of conspecifics are examined and discussed in this chapter. Finally, the settlement cue contained in the oyster shell extract was partially purified.

In chapter IV, the author discussed the nature of the settlement cue in conspecifics. Furthermore, the author discussed the possible role of this cue in the larval settlement. Characteristics of the extracted settlement cue and the probable involvement of N- linked oligosaccharides in the settlement of *C. gigas* larvae is also discussed. A settlement mechanism that involves the non-soluble settlement cue is proposed in this chapter. Lastly, a conclusion is made based in the results that a WGA-binding glycoprotein contained in the shell organic matrix of *C. gigas* is hypothesized to be the basis of larval settlement on conspecifics.

II. Larval Settlement of the Pacific oyster *C. gigas* in Response to Different Mollusks Shells and Extracts from Conspecifics Applied on Different Substrates.

II.1 Introduction

C. gigas larvae are known to settle on various substrates including sheets of slightly roughened PVC, layers of shell chips prepared by grinding clean oyster shells, bags or strings of aged clean oyster shells and various plastic or ceramic materials coated with lime/mortar mix (Helm et al, 2004). Scallop shells are also commonly used in Korea (Byung et al, 1988) and Japan (Noda 1980). Diederich (2005) demonstrated that *C. gigas* larvae show preference to shells of its own species as a settlement substrate. This observation has been interpreted as the gregarious behavior in oysters.

In this chapter, the author investigated the settlement inducing activities of shell chips (SC) prepared from 11 different molluscan species. The purpose was to check whether *C. gigas* larvae respond differently to SC of different species. The settlement inducing activity of SC prepared from shells of conspecifics was also investigated after treatments with antibiotics and heat. Settlement inducing activities of extracts of conspecific oyster shells prepared using different solvents were also evaluated. Finally, larval settlement of *C. gigas* on different substrates as-is and on substrates coated with a *C. gigas* shell extract were investigated.

II.2 Materials and Methods

II.2.1 Spawning and Larval Culture

Adult *Crassostrea gigas* used for spawning were purchased from Konagai Fisheries Cooperative (Nagasaki, Japan). These oysters were from an oyster culture farm in Konagai-cho, Nagasaki, Japan (32°55'08"N, 130°11'42"E). These were maintained in an aquarium inside the laboratory and were fed daily with a combination of *Chaetoceros gracilis* and an artificial feed for bivalves (M1, Nosan Corp., Yokohama, Japan). During the spawning season, adults were kept at 20±1°C. This was to suppress natural spawning of the broodstock. During the winter season, adults were maintained at 25±1°C to allow gonad development and maturation. Thirty different broodstock groups were used for spawning to obtain larvae for the settlement assays conducted during the period between 2008 and 2013.

Gonads were stripped from the oysters to collect gametes (Allen and Bushek 1992). Eggs and sperm were separately suspended in 2L glass beakers containing GF/C (Whatman glass fiber filter; pore size: 1.2 µm) filtered seawater (FSW) adjusted to 24°C. Eggs were washed several times with FSW through repeated decantation and were then fertilized with a small volume of the sperm suspension. Thirty minutes after artificial fertilization, fertilized eggs were collected in a 20µm net, washed four to five times with FSW and re-suspended in 2 L glass beakers containing FSW. Fertilized eggs were kept at 24±1°C in an incubator for 24 h.

After 24 h, swimming straight-hinged larvae were collected in a 40 µm net, gently washed with FSW, stocked in 2 L glass beakers at an initial density of 5 larvae mL⁻¹ and cultured in a water bath at 24±1°C. Larvae were fed daily with the following algal diet: *Chaetoceros calcitrans* (5x10⁴

cells mL⁻¹) from the 1st to the 5th day of culture, *C. calcitrans* (2.5x10⁴ cells mL⁻¹) and *C. gracilis* (2.5x10⁴ cells mL⁻¹) from the 6th to the 10th day of culture, and *C. gracilis* (5x10⁴ cells mL⁻¹) from the 11th day onward during the culture period. Cultures were aerated (20 mL min⁻¹) and the water renewed daily throughout the culture period. Cultures were kept in a dark environment but were exposed to light daily for approximately 1 h during water changing. Salinity of seawater used was 32 psu. Larvae usually reached the pediveliger stage on the 17 to 18 days after fertilization. Pediveligers employed in assays were between 24 and 28 days old after fertilization and ranged from 300 to 320 μ m in shell length.

II.2.2 Larval Settlement Assays

Twenty pediveliger larvae were released into each petri dish (\varnothing 60 mm x 15 mm height) containing 20 mL FSW and the substrate (shell chips (SC), GF/C filter paper (\varnothing 47mm, GF/C), plaster plate and nitrocellulose circles). Settlement inducing activities of the different substrates were evaluated by the number of individuals that metamorphosed to post larvae within 24 h. Post larvae were confirmed under the microscope as individuals that secreted cement substance or those with post-larval shell growth. Petri dishes, each containing 20 pediveligers and 20 mL of FSW only or with GF/C as a substrate were set as the blank control. All assays were conducted in a dark environment at 24 \pm 1 $^{\circ}$ C in an incubator.

In assays with SC of the 11 species of mollusks, tests were conducted at 0, 10, 50 and 100 mg dry weight of SC for each species. SC of *C. gigas* were further subjected to several bioassay-guided treatments using heat and antibiotic treatments.

In the assay on heat treatment of *C. gigas* SC, the amounts tested were 50 mg and 500 mg per petri dish. In the assay on antibiotic treatment of *C. gigas* SC, the amount tested was 50 mg per petri dish.

In assays to investigate the activities of *C. gigas* SC extracts (aqueous [Aq-ex], hydrochloric acid [HCl-ex], ethanol [EtOH-ex] and diethyl ether [Et₂O]) amounts assayed were expressed in weight equivalent of SC, where 1 mg SC was equivalent to 0.5 μ g of protein as determined after measuring using the Lowry assay (Lowry et al. 1951).

The extracts were applied to GF/C (\varnothing 47 mm), dried and taped to the bottom of each petri dish using double-faced adhesive tape. For each type of SC and extracts, at least six replicate experiments were conducted using larvae from at least two separate culture batches.

In the assays to find a suitable substrate for settlement assays with FD HCL-ex, different substrates as-is and with the FD HCl-ex were investigated. Substrates tested were plaster plate (35 mm x 25 mm x 2 mm, Iwao Jiki Kogyo Co. Saga, Japan), nitrocellulose membrane paper (35 mm x 25 mm, Pall Corp. USA) and GF/C filter paper (\varnothing 47 mm, Whatman International Ltd. UK). Plaster plates were first boiled in DW for 1 h to remove possible impurities and then dried in the oven at 160°C prior to use. Prior to use in assays, nitrocellulose membrane papers were washed with DW and then air dried, while GF/C filter papers were washed with DW and then dried at 160°C. FD HCl-ex was applied to the substrates at concentrations of 0, 50 and 100 mg SC equivalent per substrate, but for GF/C, HCl-ex concentration was tested until 150 mg SC equivalent. The substrates were then dried at 37°C and placed inside the petri dish. Nitrocellulose membrane papers and GF/C filter papers were each fixed to

the bottom of the petri dish with a double-faced adhesive tape while the plaster plate was just placed on the bottom of the petri dish before petri dishes were filled with 20 ml of FSW each. FD HCl-ex was also directly applied to the bottom of petri dishes at concentrations of 0, 50 and 100 mg SC equivalent per petri dish (as glass substrate), after which the petri dishes were dried at 37°C before these were each filled with 20 mL of FSW.

In assays with *C. gigas* SC extracts, 50 mg of *C. gigas* SC per petri dish was set as a positive control. In assays to test different substrates petri dishes with 50 mg and 100 mg of *C. gigas* shell chips (SC) were set.

II.2.3 Preparation of Shell Chips (SC) of the 11 Molluscan Species and Treated (heat and antibiotics) C. gigas SC

SC of the 11 species of mollusks were prepared using shells taken from living specimens. The taxonomic classifications and origins of specimens used to obtain the shells are described in Table 2.1. Prior to the preparation of SC of each species, shells were scrubbed using a metal wire brush, to remove attached organisms and visible traces of muscle tissues, washed and then dried. Dried shells were crushed using a mallet and crushed shell fragments were sieved through two metallic mesh screens; first, 1.0 mm mesh size screen, and then the 0.5 mm screen. Shells fragments that remained on the 0.5 mm mesh screen were collected and used as SC. SC were stored in a freezer at -40°C until these were used in experiments.

In the heat treatment experiment, *C. gigas* SC heated at 100°C and 200°C for 1 h and at 300°C for 3 h in an electrical oven were prepared and used in assays.

In the antibiotic treatment experiment, *C. gigas* SC were soaked in 1AB and 10AB concentrations of antibiotic solutions for 48 h following the method used by Yang et al. (2007) for sterilization of macroalgae. Antibiotic treated SC were rinsed six times using a total volume of 2 L FSW prior to use in assays. Concentrations of the drugs in 1AB of antibiotic solution were: 20 mg L⁻¹ of streptomycin sulphate, 10 mg L⁻¹ of penicillin G, 2 mg L⁻¹ of neomycin and 10 mg L⁻¹ of kanamycin, and concentrations were increased ten-fold in the 10AB solution.

Table 2.1 Taxonomy of the 11 species of mollusks used as specimens

Class	Order	Family	Species	Specimen origin
Bivalvia	Ostreoida	Ostreidae	<i>Crassostrea gigas</i>	Konagai fishing port, Nagasaki, Japan
		Ostreidae	<i>C. nippona</i>	Nagasaki Pref. Institute of Fisheries, Nagasaki, Japan
		Ostreidae	<i>C. iredalei</i>	Manila Bay, Philippines
		Ostreidae	<i>Ostrea circumpicta</i>	Hiroshima Prefectural Technology Research Institute, Hiroshima, Japan
		Ostreidae	<i>O. denselamellosa</i>	Oita Prefectural Agriculture, Forestry and Fisheries Research Center, Oita, Japan
		Ostreidae	<i>Saccostrea kegaki</i>	Koebaru, Nagasaki, Japan
		Ostreidae	<i>S. mordax</i>	Koebaru, Nagasaki, Japan
		Pectinoida	Pectinidae	<i>Patinopecten yessoensis</i>
Pterioida	Pteriidae	<i>Pinctada fucata martensii</i>	Nagasaki Pref. Institute of Fisheries, Nagasaki, Japan	
	Pinnidae	<i>Atrina pinnata</i>	Nagasaki Pref. Institute of Fisheries, Nagasaki, Japan	
Gastropoda		Haliotidae	<i>Haliotis discus</i>	Nagasaki Pref. Institute of Fisheries, Nagasaki, Japan

II.2.4 Preparation of C. gigas SC Extracts

Aqueous extract (Aq-ex) Ten grams of *C. gigas* SC was placed in a mortar and ground while adding small amounts of distilled water (DW) warmed to 60°C into the mortar. The supernatant was collected and the procedure repeated until a total of 100 mL of the supernatant was collected. The supernatant was filtered through GF/C and then used in assays. After extraction with DW, residues were collected on a glass petri dish, completely dried in an oven at 50°C and then used in assays.

Ethanol extract (EtOH-ex) Ten grams of *C. gigas* SC was soaked overnight in 100 mL of ethanol. The supernatant was then collected, filtered through GF/C and concentrated to 1 g mL⁻¹ SC equivalent using a rotary evaporator. Concentrated EtOH-ex was then applied to GF/C, air-dried and used in assays. After extraction with ethanol, residues were also collected on a glass petri dish, air-dried at room temperature for approximately 2 h until the solvent was completely evaporated. Dried residue was then used in assays.

Diethyl ether extract (Et₂O-ex) Diethyl ether extract was prepared in the same manner as EtOH-ex. After extraction with diethyl ether, residues were also collected on a glass petri dish and air-dried at room temperature. Dried residue was then used in assays.

Hydrochloric acid extract (HCl-ex) A total amount of 150 g of *C. gigas* SC was completely dissolved in 1L of 2 N HCl (Akiyama 1978). This solution was centrifuged at 12859 x g for 20 min at 4°C, the supernatant was collected and filtered through GF/C and then dialyzed against 0.01N HCl solution at 4°C for a period of three days until the final pH of the extract was 6. The hydrochloric acid extract was stored in the freezer until

used in assays. Freeze dried samples of the acid extract were also prepared and stored as powder in the freezer until used in assays and was further called oyster shell extract (FD HCl-ex). Activities of extracts were examined as described in the larval settlement assays.

Ethanol, diethyl ether and HCl were purchased from Wako Pure Chemical Co. (Osaka, Japan).

II.2.5 Statistical Analysis

Settlement inducing activities of the different amounts and species of SC, extracts and substrates were evaluated by the number of post larvae that settled and percentages were presented as arithmetic means with standard deviations (SD). Data were analyzed using binomial generalized linear models (GLM) or quasi-binomial GLM when a model resulted in overdispersion. Post hoc Tukey HSD multiple comparison test was conducted to assess differences in settlement inducing activities between samples in the model. Wald test (Draper and Smith 1998) was used for pairwise comparisons. In the analysis for settlement inducing activities of SC of the different molluscan species at 10, 50 and 100 mg the model included species and weight of SC as variables, and analysis of variance (ANOVA) was performed to check for interaction effects between these two factors. Estimated settlement differences by percentage for SC of each of the 10 species of mollusks compared to *C. gigas* SC were calculated based on odds ratios derived from the model results. All statistical analysis were carried out using the statistical package R (R Foundation for Statistical Computing: <http://www.r-project.org>, ver. 3.0.1) Differences were considered significant at $p < 0.05$.

II.3 Results

The percentage of post larvae of *C. gigas* on clean petri dishes with FSW (blank control) was $1.1 \pm 4.3\%$.

II.3.1 Settlement Inducing Activities of SC of the 11 Species of Mollusk

Percentages of *C. gigas* post larvae at 0, 10, 50 and 100 mg SC prepared from 11 different species of mollusks are as shown in Fig. 2.1. Settlement inducing activities of the different SC were assessed with Tukey HSD test for multiple comparisons after all data were fitted in the quasi-binomial model. Of the 11 different species, SC of *P. yessoensis* and *A. pinnata* did not induce settlement of *C. gigas* larvae at all weights tested. On the other hand, SC of *S. mordax*, *O. circumpecta*, *O. denselamellosa*, *P. fucata martensii* and *H. discus* induced larval settlement only at 100 mg, while *C. iredalei* and *S. kegaki* induced settlement at 50 and 100 mg of SC. Percentages of post larvae on SC of *C. gigas* and *C. nippona* were significantly higher than those of the blank control at all weights tested (Fig. 2.1). For species that induced larval settlement of *C. gigas*, percentages of post larvae increased with the weight of SC used and were highest at 100 mg. Analysis of variance also showed that the settlement inducing activity of SC was significantly affected by both species ($p < 0.0001$) and weight ($p < 0.0001$) (Table 2.2). However, the interaction between species and weight of SC did not significantly improve the model ($p = 0.2566$) (Table 2.2) and hence, not included as a variable in the final GLM model.

Estimated settlement differences (%) of each of the 10 species of mollusks compared to *C. gigas* were calculated from the final GLM model and plotted in Fig. 2.2. Of the 10 species of SC, only *C. nippona* showed a settlement inducing activity that was not significantly different from that of

C. gigas. By contrast, SC of the other nine species all showed lower settlement inducing activities, and estimated settlements on SC of these species were 60 to 95% less than that of *C. gigas*. Except for *P. fucata martensii*, estimated settlements of *C. gigas* larvae on SC of species not belonging to Ostreidae were 90% less than that of conspecific SC (Fig. 2.2).

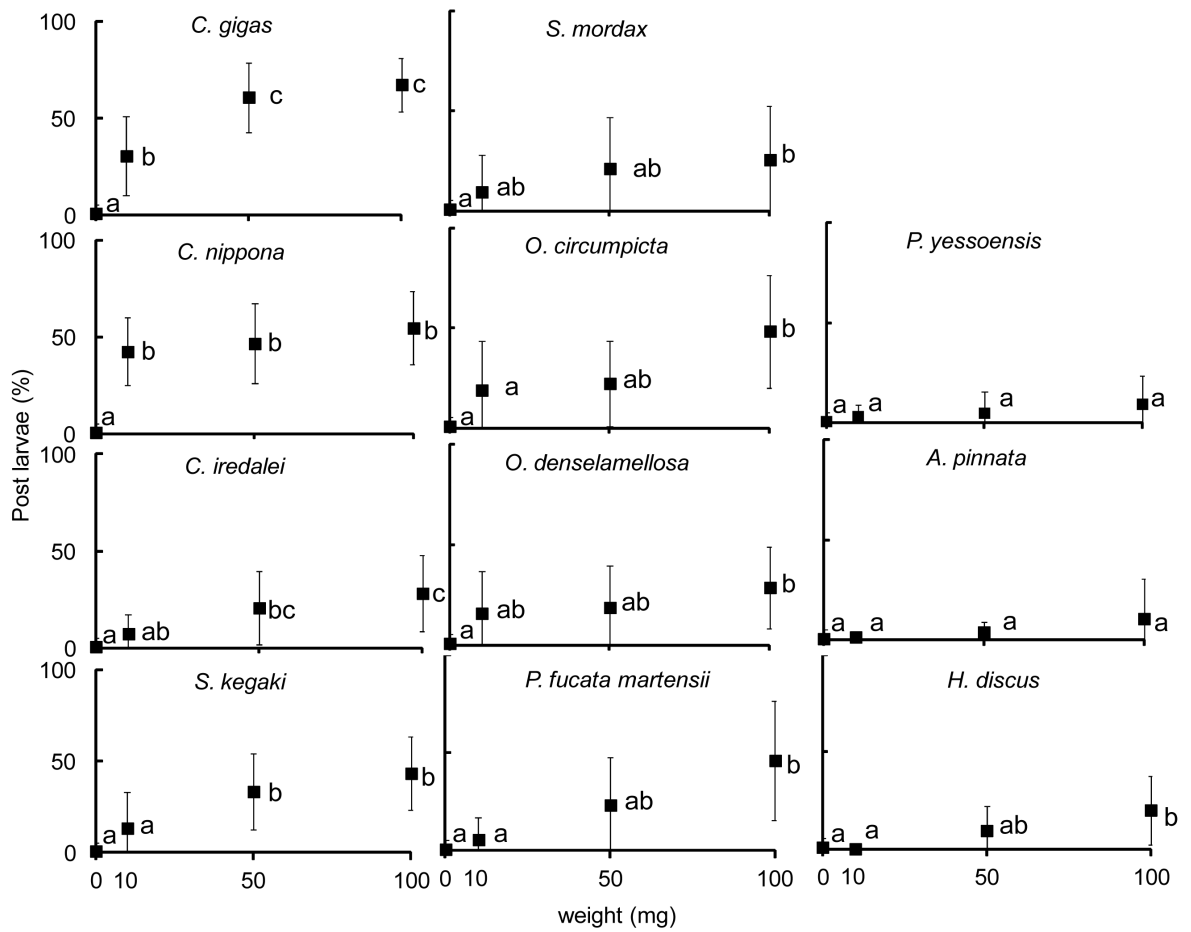


Fig. 2.1 Percentages of post larvae that settled on SC of the different species of mollusks.

Closed squares are means of 6 to 30 replicates and error bars represent standard deviation (SD). Letters indicate results of the post hoc Tukey HSD test of activities within species at 0, 10, 50 and 100 mg. Values connected by the same letter are not significantly different ($p \geq 0.05$).

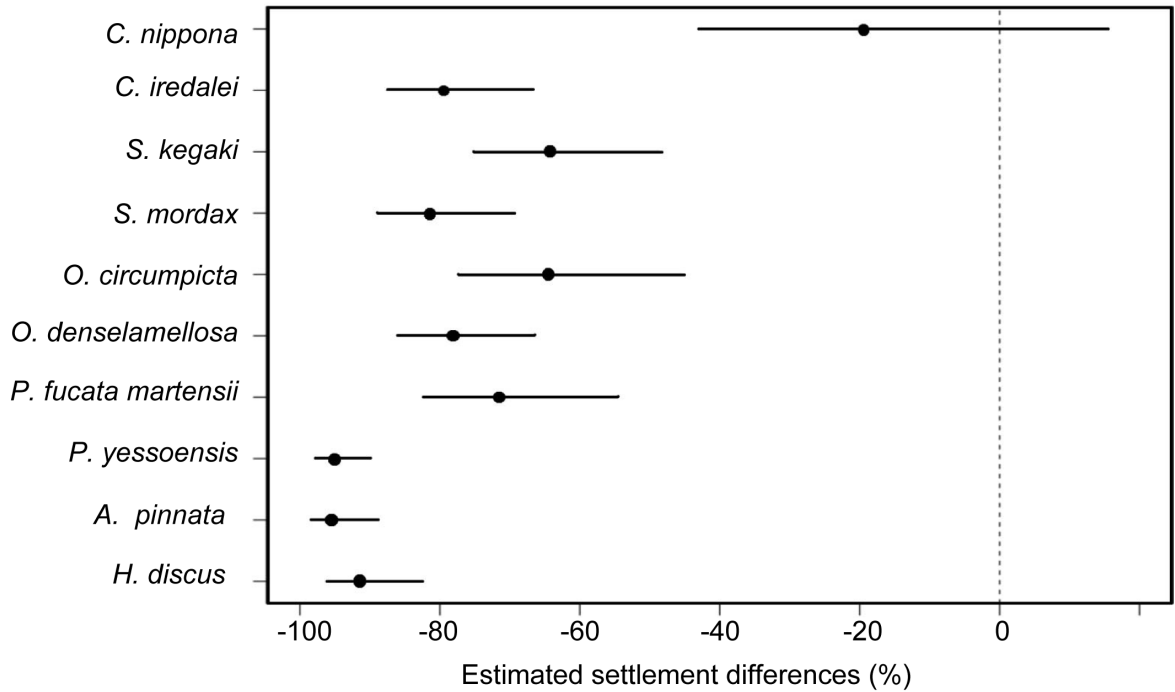


Fig. 2.2 Estimated percent settlement differences of the 10 molluscan species SC vs. *C. gigas* SC.

Closed circles represent mean estimates and error bars are 95% confidence intervals. Estimated settlement differences between the ten molluscan species and *C. gigas* (as the reference) were calculated based on the odds ratios.

Table 2.2 ANOVA result of the effect of species and amount of SC on *C. gigas* larval settlement

	Df	Deviance	Residual Df	Residual Deviance	p
Null (intercept)			383	3149.4	
Species	10	1041.65	373	2107.8	<0.0001
Amount	1	383.33	372	1724.4	<0.0001
Species x Amount	10	53.13	362	1671.3	0.2566

Statistics of the quasi-binomial GLM applied to the output for dependent variable larval settlement. Species refers to the 11 species of SC tested; amount refers to the weight of SC.

II.3.2 Effect of Heat and Antibiotics Treatments on the Activity of C. gigas SC

The effect of heat treatments for 1 h and 3 h on the activity of *C. gigas* SC is shown in Fig. 2.3A. Tukey HSD test showed that up to 200°C, heat had no effect on the settlement inducing activity of *C. gigas* SC, which remained constant regardless of the heating time (1 h and 3 h) and the amount of SC (50 mg and 500 mg) heated. However, the activity of 500 mg SC significantly decreased after heating at 300°C for 3 h. No difference in activity was also found between 50 mg SC heated at 200°C for 1 h and 500 mg SC heated at 300°C for 3 h. The effect of antibiotics treatment on the activity of *C. gigas* SC is shown in Fig. 2.3B. Tukey HSD test also showed that treatment with 1AB and 10AB concentrations of antibiotics solution for 48 h did not affect the activity of *C. gigas* SC.

II.3.3 Settlement Inducing Activities of C. gigas SC Extracts

The settlement inducing activities of Et₂O-ex, EtOH-ex, Aq-ex, HCl-ex and FD HCl-ex are shown in Fig. 2.4. Percentages of post larvae in Et₂O-ex, EtOH-ex and Aq-ex at all concentrations tested were <8% at the highest, and were not significantly different from the blank control as assessed in the Tukey HSD test. The percentage of post larvae on the SC residue of diethyl ether extraction was 62% and was at the same level as the positive control (50 mg SC). On the other hand, settlement inducing activities of SC residues of ethanol and water extractions decreased as compared to the positive control, and their percentages of post larvae were 40% and 41%, respectively, indicating that the extraction process significantly changed the activity of SC.

HCl-ex and FD HCl-ex showed high settlement inducing activities at all concentrations tested; percentages of post larvae were all significantly higher than the blank control and were at the same level as the positive control (50 mg SC), except for the 50 mg SC eq of HCl-ex. Freeze drying did not also affect the activity of HCl-ex; no difference was observed in the percentages of post larvae between HCl-ex and FD HCl-ex at any concentration (Fig. 2.4).

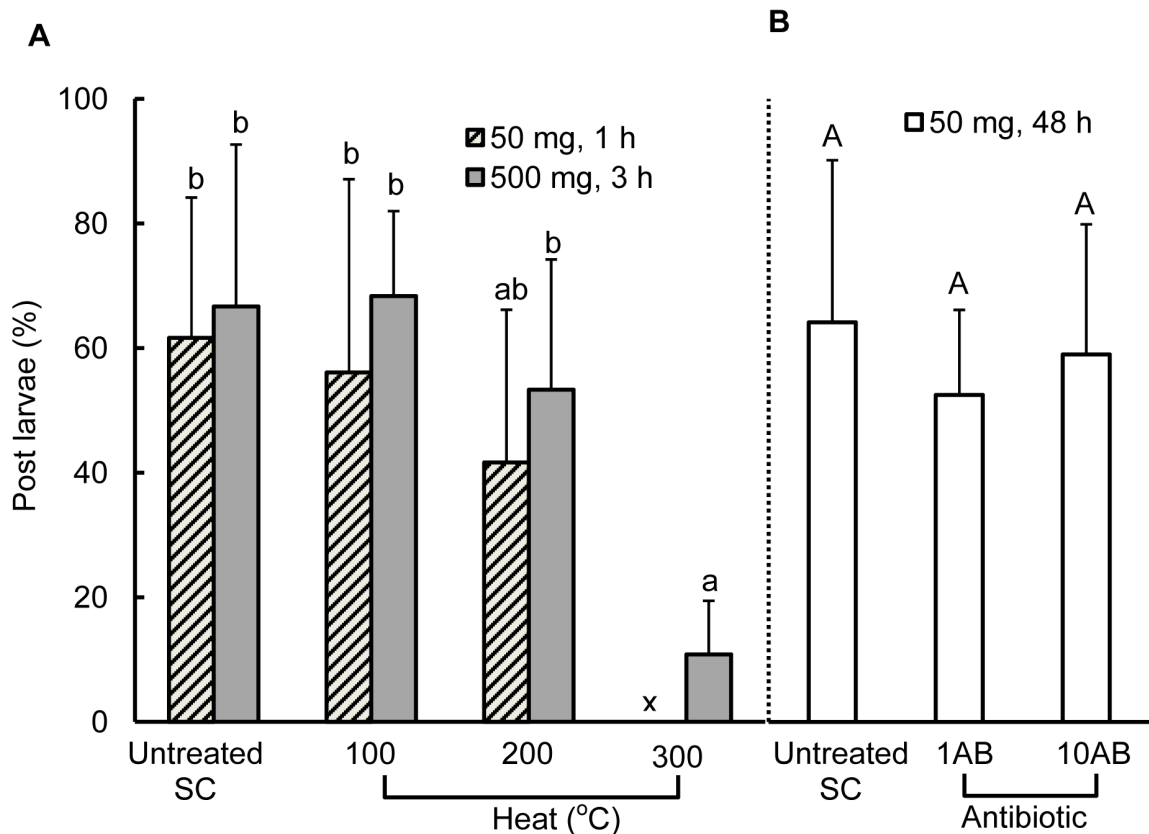


Fig. 2.3 (A, B) Percentages of post larvae on *C. gigas* SC that were heated at different temperatures (A), and treated with antibiotic solutions (B).

A: Boxes with slanting lines and shading indicate results of heat treatment experiments of 50 mg SC and 500 mg SC, respectively. x indicates no data. B: blank boxes indicate the antibiotic treatment experiment of 50 mg SC. 1AB = streptomycin sulphate 20 mg L⁻¹, penicillin G 10 mg L⁻¹, neomycin 2 mg L⁻¹, and kanamycin 10 mg L⁻¹, and 10AB = 1AB concentration increased tenfold. Data are means of 6 to 9 replicates and error bars represent standard deviations (SD). Lowercase and uppercase letters indicate results of the post hoc Tukey HSD test on heat and antibiotic treatments, respectively. Groups connected by the same letter are not significantly different ($p \geq 0.05$).

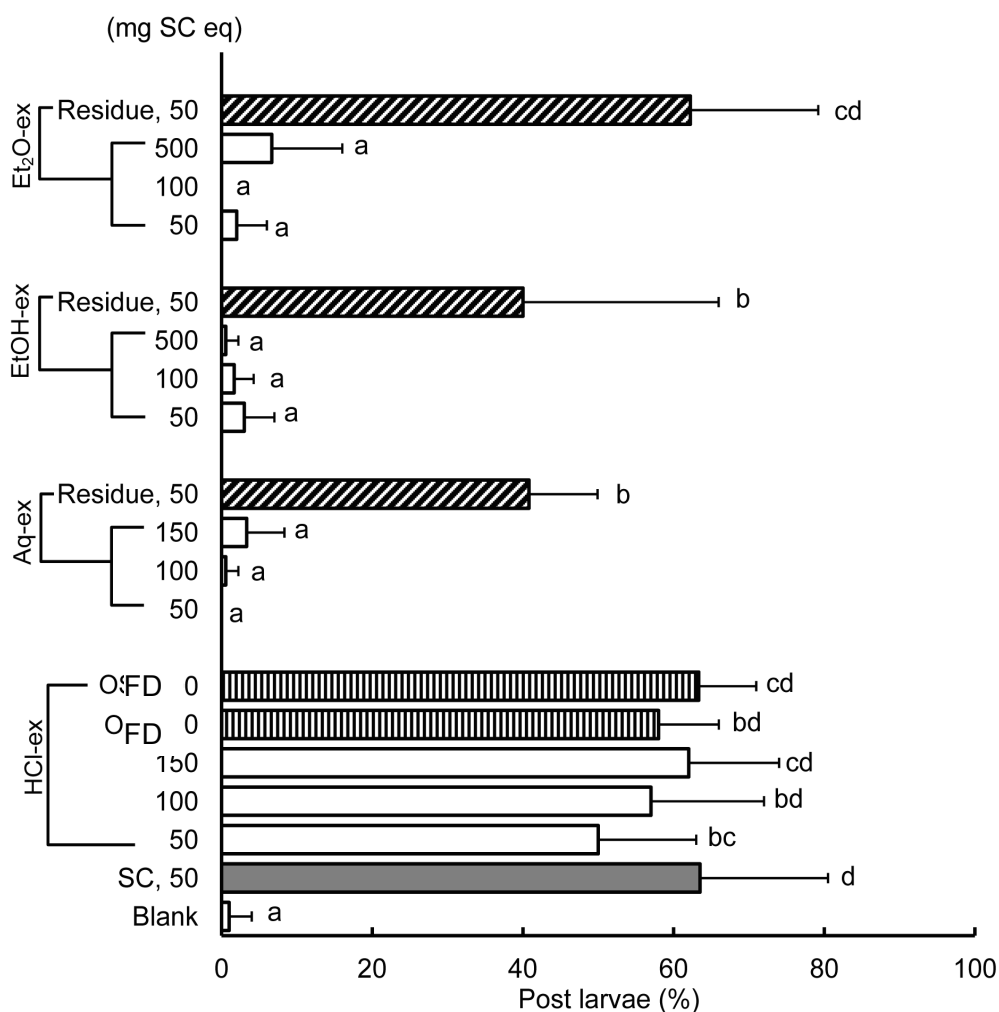


Fig. 2.4 Percentages of post larvae on Et₂O-ex, EtOH-ex, Aq-ex, HCl-ex and residues after extraction of *C. gigas* SC.

Amounts assayed are expressed in mg equivalent of SC (mg SC eq), where each weight equivalent of the extract corresponds to the amount of SC extracted. Et₂O-ex, EtOH-ex, Aq-ex and HCl-ex indicate *C. gigas* SC extracts with diethyl ether, ethanol, water and hydrochloric acid, respectively. FD HCl-ex indicates freeze dried HCl-ex. Data are means of 6 to 15 replicates and error bars represent standard deviations (SD). Letters indicate results of the post hoc Tukey HSD test. Groups connected by the same letter are not significantly different ($p \geq 0.05$).

II.3.4 C. gigas Larval Settlement on Different Substrates as-is and with FD HCl-ex.

Settlement of *C. gigas* larvae on SC and on different substrates with and without FD HCl-ex is as shown in Fig. 2.5. Percentage of post larvae on 50 mg of SC was 63% but larval settlement (67%) did not increase even when the amount of SC was doubled to 100 mg. When *C. gigas* larvae were exposed to glass (no additional substrate placed in the petri dish), nitrocellulose, plaster and GF/C in the absence of FD HCl-ex, larvae remained swimming and did not settle after 24 h, indicating that these four substrates were inert and alone do not induce larval settlement nor did these exhibit toxicity since no mortality was observed. However, larval settlement was observed in all cases when FD HCl-ex were absorbed at different concentrations to the three substrates and when applied directly to glass. In GF/C with FD HCl-ex, percentages of post larvae were >50% at all the concentrations tested and increasing the amount of FD HCl-ex in the GF/C did not significantly increase the percentages of post larvae. Moreover, the percentages of post larvae on GF/C with 50 and 100 mg SC eq of HCl-ex were at the same levels as those exposed to 50 and 100 mg SC, respectively ($p>0.05$). By contrast, percentages of post larvae in nitrocellulose and plaster containing FD HCl-ex at 50 and 100 mg SC eq, respectively, were at the same level as their respective controls without the FD HCl-ex ($p>0.05$). Direct application of FD HCl-ex to glass resulted in larval settlement on the glass and settlement at 100 mg SC eq was at the same level as that on 100 mg SC and GF/C containing FD HCl-ex ($p>0.05$). Latter assays were therefore conducted using GF/C as the substrate for FD HCl-ex.

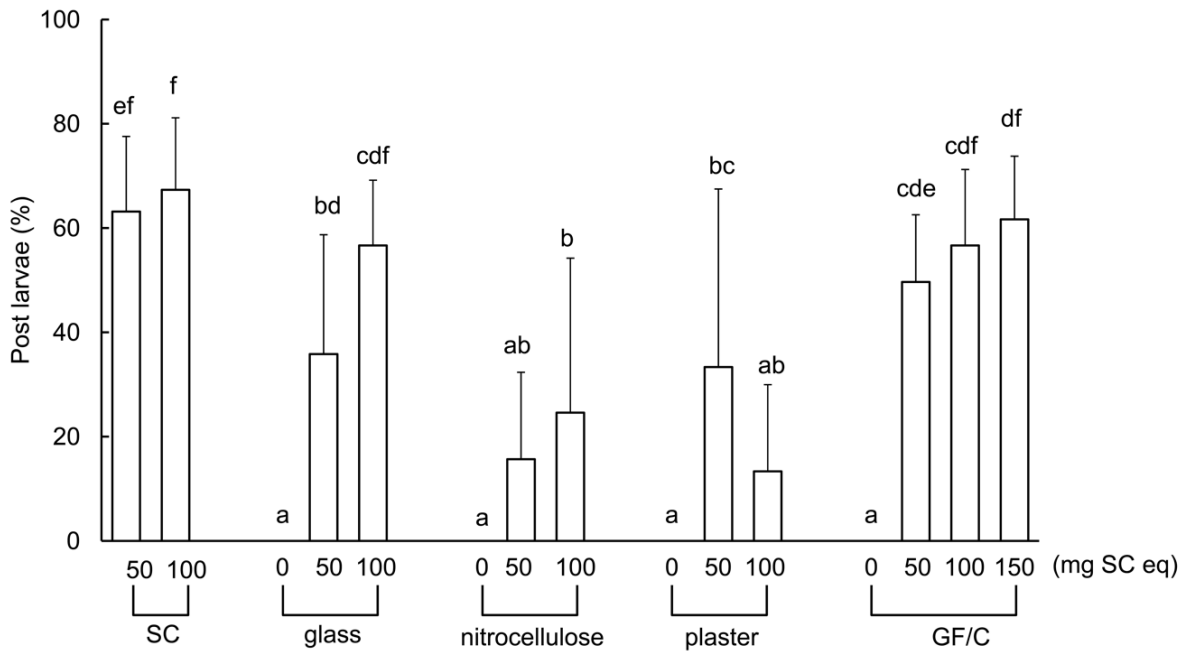


Fig. 2.5 Percentages of *C. gigas* post larvae on conspecific shell chips (SC) and on different substrates without FD HCl-ex and those containing FD HCl-ex

Amounts of FD HCl-ex in the substrates are expressed in mg equivalent of SC (mg SC eq) where 1 mg SC was equivalent to 0.5 μ g protein of FD HCl-ex. Data are means of 6 to 30 replicates and error bars represent standard deviations (SD). Letters indicate results of the post hoc Tukey HSD test. Groups connected by the same letter are not significantly different ($p \geq 0.05$).

II.4 Discussion

In this chapter, the author demonstrated that larvae of the Pacific oyster *C. gigas* differentially settle on shells of different species of mollusks (Fig. 2.1), albeit in higher numbers on conspecific shells and those of *C. nippona* (Fig. 2.2). This finding suggests that larvae may recognize shells of their own or related species during settlement and will respond favorably by settling in high numbers. This is consistent with field observations of Diederich (2005) where recruitment of *C. gigas* larvae was higher on shells of conspecifics than those of *Mytilus edulis*. Diederich (2005) interpreted this observation as the difference in the texture of shells and that larvae exhibited preference for rough oyster shells over smooth shells of mussels. Crisp (1967), however, suggested that chemical cues in shells maybe involved since *C. virginica* larvae settled almost entirely on conspecific shells but the destruction of the organic layers on the surface of shells clearly made the substratum unfavorable. In species that induced *C. gigas* larvae to settle, larval settlement was proportionate to the amount of SC given to the larvae (Fig. 2.1). Thus, increasing the amount of SC also increases the likelihood of larvae to detect the settlement cue, resulting in higher settlement of larvae where amounts of SC were increased.

Most molluscan shells consist of a mineral portion that is more than 95% of the weight and an organic fraction that is less than 5% (Marin and Luquet 2005). *C. gigas* shells contain more than 99% calcium carbonate and 0.5%, by weight, of some organic matrices (Marie et al. 2011). These organic matrices in mollusks shells, which consist of polysaccharides and proteins, show great diversity in their molecular weights and amino acid compositions among species, and their secretion is fundamentally controlled by genes involved in shell formation (Kobayashi and Samata

2006). The high settlement response of *C. gigas* larvae to shells of its own species and *C. nippona* suggests that larvae may recognize a settlement cue that may be abundant in conspecific shells and closely related species. In species with settlement inducing activities that were lower than *C. gigas* and *C. nippona*, the settlement cue may be present in the shells in smaller amounts. Thus, increasing the amount of shell given to the larvae can improve settlement inducing activity, as explained earlier in this discussion. Another explanation for the lower settlement response of *C. gigas* larvae to other species may be the difference in the matrix component of the shells. We are currently investigating the shell matrix compositions of the different shell species in order to check for possible relationships between shell matrix components and settlement inducing activities of different shell species.

The putative settlement cue in shells of conspecifics was stable even at 200°C and decreased in activity only when it was charred at 300°C (Fig. 2.3A). Knight-Jones (1953) heated pieces of slates covered with bases of newly detached barnacles and observed that the activity of the settlement cue from *Balanus balanoides* shells remained unaffected until 200°C but this was gradually destroyed at about 250°C. The stability of some proteins from the shell matrix at high temperatures has also been demonstrated elsewhere (Totten et al. 1972; Akiyama 1978). The settlement inducing activity of *C. gigas* SC also remained intact even after antibiotics treatment (Fig. 2.3B). Heat (Bao et al. 2007a) and antibiotics (Kirchman et al. 1982a; Satuito et al. 1995; Unabia and Hadfield 1999; Lau and Qian 2001; Bao et al. 2007a) treatments have been used to kill constituent organisms in microbial biofilms, particularly bacterial constituents in the latter treatment. Consequently, these treatments resulted in the loss of biofilm and/or

bacterial activity to induce larval settlement. The fact that *C. gigas* SC retained settlement inducing activity even after heat and antibiotic treatments suggests that the settlement cue was contained in *C. gigas* shell itself and distinct from the cue in bacterial biofilms reported in early studies (Fitt et al. 1989).

Aqueous and organic solvent extracts of *C. gigas* shells did not significantly induce larval settlement at all concentrations tested, indicating that the chemical cue was not efficiently extracted by these solvents. By contrast, the HCl extract of *C. gigas* shells showed settlement inducing activity that was equivalent to the activity of the shell itself (Fig. 2.4). Therefore, HCl proved to be the most efficient solvent to use in extracting the settlement inducing compound in *C. gigas* shells. SC residues obtained after water and ethanol extractions exhibited lower activities as compared to SC (positive control) (Fig. 2.4), and this finding suggests the possibility of the presence of a settlement inducer compound(s) in both Aq-ex and EtOH-ex, although concentrations of Aq-ex and EtOH-ex tested in this investigation may not have been enough to significantly induce larval settlement. Moreover, during water extraction of SC, SC are crushed to a powdered state and therefore, this change in the physical property may also account for the decrease in settlement inducing activity of the residue obtained. Nevertheless, larval responses to Aq-ex, EtOH-ex and Et₂O-ex warrant further investigation.

Acids are often used in the demineralization of shells to collect the protein matrix (Totten et al. 1972; Akiyama 1978; Mann et al. 2012). Results indicate that the acid-soluble matrix of *C. gigas* shells contained a settlement inducing compound for conspecific larvae.

To characterize the settlement inducer from conspecifics it is important to use a suitable substrate for the settlement assays. A suitable substrate should be inert and is not repelled by the larva being studied, and at the same time is easy to handle or manipulate. We tested larval settlement behavior of *C. gigas* on plaster plates, nitrocellulose membrane, GF/C filter papers and glass (Fig. 2.5) We found that all substrates tested were inert and as is, did not induce larval settlement; all larvae remained swimming after 24 h. On the other hand, adding the FD HCl-ex to these substrates resulted in larval settlement on all substrates, although larvae settled differentially on the different substrates, settling in highest numbers on GF/C filter papers and glass (Fig. 2.5). The low settlement on plaster plates containing FD HCl-ex may be explained by the fact that it was thicker (ca. 2 mm thickness) than the other substrates and therefore FD HCl-ex absorbed inside the plaster plate may not have been available to the larvae. Nitrocellulose membrane, which absorbs protein, also showed lower settlement than the GF/C filter paper. Nitrocellulose membrane had an apparently smoother surface than GF/C filter paper, and this may have affected larval settlement on nitrocellulose membrane, although this warrants further investigation. Matsumura et al. (1998a) successfully used nitrocellulose membranes as substrates for barnacle larval settlement. In the case of *C. gigas* larvae, however, GF/C filter papers yielded the best results when FD HCl-ex was added to it. Therefore, GF/C filter paper was chosen as the substrate for *C. gigas* larval settlement assays in this study.

In conclusion, *C. gigas* larvae settled on shells of different species of mollusks but settled in higher numbers on shells of its conspecifics and *C. nippona*, suggesting that the cue may be more abundant or in a more available form to the larvae in shells of conspecifics and *C. nippona* than in

other species. In *C. gigas* shells, the settlement cue was heat stable and was extracted only by dissolving the shell in hydrochloric acid. This settlement cue can find application in the aquaculture industry of this economically important species. For example, this insoluble cue can be used to develop effective spat collectors of both wild and hatchery grown seeds by applying it on surfaces.

III. Characterization and Partial Purification of a Settlement cue from Shells of Conspecifics that Induces Larval Settlement in the Pacific Oyster *C. gigas* Larvae.

III.1. Introduction

The identification of settlement inducing compounds of various marine invertebrates has been actively pursued in the field of larval biology these recent years. In the previous chapter (Chapter II) the author found that *C. gigas* larvae settled in higher numbers on shells of its conspecifics. The settlement cue in *C. gigas* shells was heat stable and was extracted only by dissolving the shell in hydrochloric acid (FD HCl-ex).

The inducing effect of conspecifics on larval settlement has also been studied for various oyster species i.e., *O. edulis* (Knight-Jones 1939; Bayne 1969), *Crassostrea virginica* (Crisp 1967; Hidu 1969; Veitch and Hidu 1971; Keck et al. 1971; Tamburri et al. 1996), *C. gigas* (Hirata 1998; Hirata 2005; Tamburri et al. 2007), *C. ariakensis* (Tamburri et al. 2008). In *C. virginica*, adult shells, water preconditioned by adults and ‘oyster shell liquor’ have been shown to promote larval settlement (Crisp 1967; Keck et al. 1971; Tamburri et al. 1992). Veitch and Hidu (1971) further reported that the inducing substance present in the ‘oyster shell liquor’ was a thyroxine containing protein with MW greater than 100,000 Da. Crisp (1967) observed that larvae of *C. virginica* settled almost entirely on conspecific shells but destruction of the organic layers on its surface clearly rendered the substratum unfavorable. The pretreatment of scallop shell spat-collectors with seawater containing live adult oysters increased the ratio of larval settlement to spat collectors in *C. gigas* (Hirata 1998). *C. gigas* larvae have also been observed to settle preferentially on shells of

conspecifics than shells of mussels, and larval settlement on shells of both living and dead conspecifics were the same (Diederich 2005). Although these studies, the characterization and the identification of the settlement cue(s) responsible for the gregarious behavior in oysters remains unclear.

In the present chapter (Chapter III), the oyster shell extract (FD HCl-ex) was subjected to physical and chemical treatments. Moreover, sugar-blocking experiments using commercial lectins to investigate the role of sugar compound structures in the FD HCl-ex on larval settlement and to check whether the “larval lectin receptor model” (Kirchman 1982b; Matsumura et al. 1998a; Jouuchi et al. 2007) will also hold true for *C. gigas*.

III.2. Materials and Methods

III.2.1 Spawning and Larval Culture

Adult *C. gigas* were purchased from Konagai Fisheries Cooperative (Nagasaki, Japan) for use in spawning. These oysters were from an oyster culture farm in Konagai-cho, Nagasaki, Japan (32°55'08"N, 130°11'42"E). Broodstock were maintained in the laboratory as previously described in Chapter II section II.2.1. Three different broodstock groups were used for spawning to obtain larvae for the settlement assays.

Spawning method and larval culture condition were as described in Chapter II section II.2.1.

III.2.2 Larval Settlement Assays

Larval settlement assays were conducted either in glass petri dishes (ø 60 mm x 15 mm height) or in polystyrene multiwell plates (6-wells; ø 34 mm x 17 mm height). Twenty pediveliger larvae were released into each petri dish filled with 20 mL of FSW, while 10 larvae were released into each well plate filled with 10 mL of FSW.

Assays to test settlement in response to treated or untreated FD HCl-ex and fractions were conducted using glass petri dishes while assays on the effects of lectins and the sugar N-Acetyl-D-glucosamine (GlcNAc) were conducted using multiwell plates. For each test variable, at least six replicate experiments were conducted using larvae from at least two separate culture batches. Settlement was evaluated by the number of individuals that metamorphosed to post larvae within 24 h. Post larvae were confirmed under the microscope as individuals that secreted cement

substance or those with post-larval shell growth. All assays were conducted in a dark environment at $24 \pm 1^\circ\text{C}$ in an incubator.

III.2.3 Treatments of FD HCl-ex

Shell chips (SC) and the oyster shell extract (FD HCl-ex) of conspecifics were prepared according to the method described in Chapter II. FD HCl-ex was dissolved in distilled water to desired concentration prior to use in all treatment assays.

In the heat treatment experiment, FD HCl-ex solutions were heated at 60°C and 100°C for 30 min in a water bath, cooled and applied to the GF/C. The GF/C were then dried and taped to the bottom of petri dishes.

Pepsin (Sigma, St. Louis MO, USA) and trypsin (Wako Pure Chemical Co., Osaka, Japan) treatments of FD HCl-ex were done following the method of Jouuchi et al. (2007) with slight modifications. Pepsin was dissolved at a concentration of 1 mg mL^{-1} in diluted HCl (pH 2). A counterpart solution without pepsin was also prepared. GF/C containing FD HCl-ex (100 mg SC equivalent) were soaked in 30 mL solutions with and without 1 mg mL^{-1} pepsin for 4 h at 37°C . Trypsin was dissolved at concentrations of 1 and 10 mg mL^{-1} in phosphate buffer (pH 7.6). A counterpart solution without trypsin was also prepared. GF/C containing FD HCl-ex (100 mg SC equivalent) were soaked in 30 mL solutions with and without the trypsin for 2 h at 37°C . After the pepsin and trypsin treatments, GF/C were washed twice, first in 1 L DW and then in 1 L FSW, dried and assayed.

FD HCl-ex was also subjected to enzymatic deglycosylation with peptide-N-glycosidase F (PNGase F, Sigma-Aldrich, St. Louis MO, USA). Twenty-five μL of phosphate buffer (100 mM, pH 7.5) was added to an

equivalent volume of 200 μg of FD HCl-ex. The mixture was then incubated with 10 IU of PNGase F at 37°C for 24 h (Jouuchi et al. 2007). A counterpart solution was also prepared but without PNGase F. After 24 h, FD HCl-ex solutions with and without the PNGase F were applied to GF/C, and the GF/C washed as described above, dried and then assayed. The FD HCl-ex was also subjected to chemical deglycosylation using trifluoromethanesulfonic acid (TFMS, Wako Pure Chemicals Co., Osaka, Japan) following the manufacturer's instruction manual. Equal amounts of FD HCl-ex (0.5 mL) and TFMS (0.5 mL) were mixed and let to react for 1h on ice. After 1 h, 0.5 mL of 1M Tris was added to the solution, and was dialyzed overnight against DW to remove TFMS from the mixture. A counterpart solution was also prepared in the same manner but without TFMS. After dialysis, FD HCl-ex treated with TFMS and its counterpart solution were applied to GF/C, washed as described above, dried and then assayed.

III.2.4 Assays with Lectins and N-acetyl-D-glucosamine (GlcNAc)

Settlement inducing activities of SC (50 mg) and GF/C filter papers (\varnothing 21 mm) containing FD HCl-ex (100 mg SC eq per GF/C [FD HCl-ex paper]) were assayed in the presence of lentil lectin (LCA), concanavalin A (ConA), soybean lectin (SBA) and wheat germ agglutinin (WGA) at concentrations of 0, 0.5, 5 and 50 $\mu\text{g mL}^{-1}$. These lectins were selected based on previous literature (Kirchman 1982b; Matsumura et al., 1998a; Jouuchi et al., 2007). Lectins were added to desired concentrations into the wells, each well containing 10 mL of FSW and either 50 mg of SC or the FD HCl-ex paper, before pediveliger larvae were released into it. FD HCl-

ex papers used in the assays were dried at 37°C. LCA, ConA, SBA and WGA were purchased from Sigma-Aldrich (St. Louis MO, USA).

FD HCl-ex papers and pediveligers were treated with 0.5, 5 and 50 $\mu\text{g mL}^{-1}$ of WGA and the effect of WGA treatment on both FD HCl-ex papers and pediveligers were also investigated. Dried FD HCl-ex papers were separately immersed in 10 mL WGA solutions at concentrations of 0.5, 5 and 50 $\mu\text{g mL}^{-1}$ in FSW. After 2 h treatment, WGA-treated FD HCl-ex papers were washed three times with 1 L of FSW and used in assays as described above. Dried FD HCl-ex papers were also washed in the same manner with 1 L of FSW and used as the control. *C. gigas* pediveligers were also treated in 10 mL WGA solutions at concentrations of 0.5, 5 and 50 $\mu\text{g mL}^{-1}$ in FSW for 2 h. After 2 h, WGA-treated pediveligers were also washed three times in 1 L of FSW and subsequently 10 pediveligers were transferred into each well plate containing FD HCl-ex paper and 10 mL of FSW. Untreated pediveligers were also released into well plates containing FD HCl-ex papers and FSW as the control.

Since lectin-mediated processes can be inhibited by low molecular weight sugars (reviewed by Lis and Sharon, 1986), the effect of GlcNAc (Wako Pure Chemical Co., Osaka, Japan) on larval settlement on FD HCl-ex paper was investigated. GlcNAc was tested at 10^{-4} , 10^{-5} and 10^{-6} M concentrations by dissolving the sugar in FSW. GlcNAc is the sugar to which WGA is known to bind to (Nagata and Burger 1974). Hence, the effect of 10^{-4} , 10^{-5} and 10^{-6} M concentrations of GlcNAc on the inhibiting activity of WGA was also investigated. Dried FD HCl-ex papers were immersed in solutions containing 50 $\mu\text{g mL}^{-1}$ of WGA or mixtures of 50 $\mu\text{g mL}^{-1}$ of WGA and different concentrations (10^{-4} , 10^{-5} , 10^{-6} M) of GlcNAc

for 2 h. After 2h, treated FD HCl-ex papers were washed three times in 1 L of FSW and then used in assays. FD HCl-ex papers that were not treated with the mixture of WGA and GlcNAc were also washed in the same manner with 1 L of FSW and used as control.

III.2.5 Dyeing SC and FD HCl-ex -containing GF/C Filter Papers with Fluorescein Isothiocyanate (FITC) Conjugated WGA

Fluorescein isothiocyanate conjugated WGA (FITC-WGA) (Sigma-Aldrich, St. Louis MO, USA) was used to confirm the presence of a lectin binding settlement inducing compound in SC and FD HCl-ex. A small amount of SC was placed inside a glass petri dish and dyed with FITC-WGA diluted in DW at 0.5 mg mL^{-1} for 5 min. After 5 min, FITC-WGA dyed SC were washed with 1 L of FSW and placed on a glass slide for fluorescence microscopic observation. Observation was carried on through a GFP filter under a Carl Zeiss epifluorescence microscope (Stemi SV11, Germany). Similarly, $10 \mu\text{g}$ and $20 \mu\text{g}$ protein concentrations of FD HCl-ex were separately applied to GF/C (\varnothing 21 mm), dried at 37°C and dyed for 5 min with 0.5 mg mL^{-1} of FITC-WGA in DW. After 5 min, dyed GF/C filter papers containing FD HCl-ex were washed in 1 L of FSW. GF/C filter paper without FD HCl-ex was also dyed with FITC-WGA and washed in the same manner as above and used as the control. FITC-WGA dyed GF/C filter papers both with and without the FD HCl-ex were also subjected to fluorescence microscopic observation.

III.2.6 Gel Filtration of FD HCl-ex

FD HCl-ex powder was dissolved in milliQ filtered sterile water (MilliQ Millipore Water System, Bedford MA, USA), applied to a Superdex 200 10/300 GL column equilibrated with 0.5 M NaCl using a 500

μL loop, and eluted with the same buffer at a rate of 0.5 mL min^{-1} , using the HPLC system. A total of 35 tubes of 0.5 mL fractions were collected, pooled into 3 molecular size range fractions and then assayed. The pooled fractions were also subjected to SDS-PAGE.

III.2.7 SDS PAGE of FD HCl-ex

Protein contents of each of the FD HCl-ex and fractions were initially measured by UV absorption at 280nm (Aitken and Learmonth 1996). Next, 10 μg protein content of FD HCl-ex and its pooled fractions were each dissolved in 30 μL distilled water and then homogenized in 30 μL of sample buffer containing 125 mM tris(hydroxymethyl)aminomethane HCl (pH 6.8), 4% sodium dodecylsulfate (SDS), 10% 2-mercaptoethanol, 0.004% bromophenol blue and 10% glycerol. The homogenates were then boiled for 10 min. After centrifugation at $1744 \times g$ for 10 min, FD HCl-ex and pooled fractions homogenates were used directly for analysis by polyacrylamide gel electrophoresis containing SDS (SDS-PAGE) according to Laemmli's (1970) method. Aliquots (20 μL) of samples were electrophoresed on a separation gel containing 10% acrylamide with a stacking gel of 3% acrylamide at 20 mA for 1.5 h. Protein profiles of samples were visualized with Stains-all (Sigma-Aldrich, St. Louis MO, USA).

III.2.8 Statistical Analysis

Settlement inducing activities in all experiments were evaluated by the number of post larvae that settled and percentages were presented as arithmetic means with standard deviations (SD). Data were analyzed using binomial generalized linear model (GLM). Post hoc Tukey HSD multiple

comparison test was conducted to assess differences in settlement inducing activities between samples in the model. Wald test (Draper and Smith 1998) was used for pairwise comparisons. All statistical analysis were carried out using the statistical package R (R Development Core Team, 2006) Differences were considered significant at $p < 0.05$.

III.3. Results

III.3.1 Effect of Treatments on the FD HCl-ex Activity

Percentage of post larvae of FD HCl-ex (100 mg SC eq) prior to heating was 64%, while those after heating at 60°C and 100°C for 30 min were 56% and 62%, respectively (Fig. 3.1). Tukey HSD test revealed no difference in the settlement inducing activities between the three groups ($p \geq 0.05$).

The effects of pepsin and trypsin treatments on the activity of FD HCl-ex are as shown in Fig. 3.2. Activity of FD HCl-ex significantly decreased after treatment with 1 mg mL⁻¹ of pepsin ($p < 0.05$, Wald test); percentages of post larvae of FD HCl-ex before and after treatment with 1 mg mL⁻¹ pepsin were 35% and 3%, respectively. Activities of FD HCl-ex treated with 1 and 10 mg mL⁻¹ of trypsin also significantly decreased as compared to the control (0 mg mL⁻¹ trypsin) ($p < 0.05$, Wald test), which was FD HCl-ex subjected to the same treatment procedure but without trypsin.

The effects of deglycosylation using PNGase F and TFMS on the activity of FD HCl-ex are as shown in Fig. 3.3. The activity of FD HCl-ex after treatment with 10 IU of PNGase F significantly decreased as compared to the control (0 IU PNGase F) ($p < 0.05$, Wald test), which was FD HCl-ex subjected to the same treatment procedure but without PNGase F. The activity of FD HCl-ex after treatment with 0.5 mL of TFMS also significantly decreased as compared to the control (0 mL TFMS) ($p < 0.05$, Wald test), which was FD HCl-ex subjected to the same treatment procedure but without TFMS.

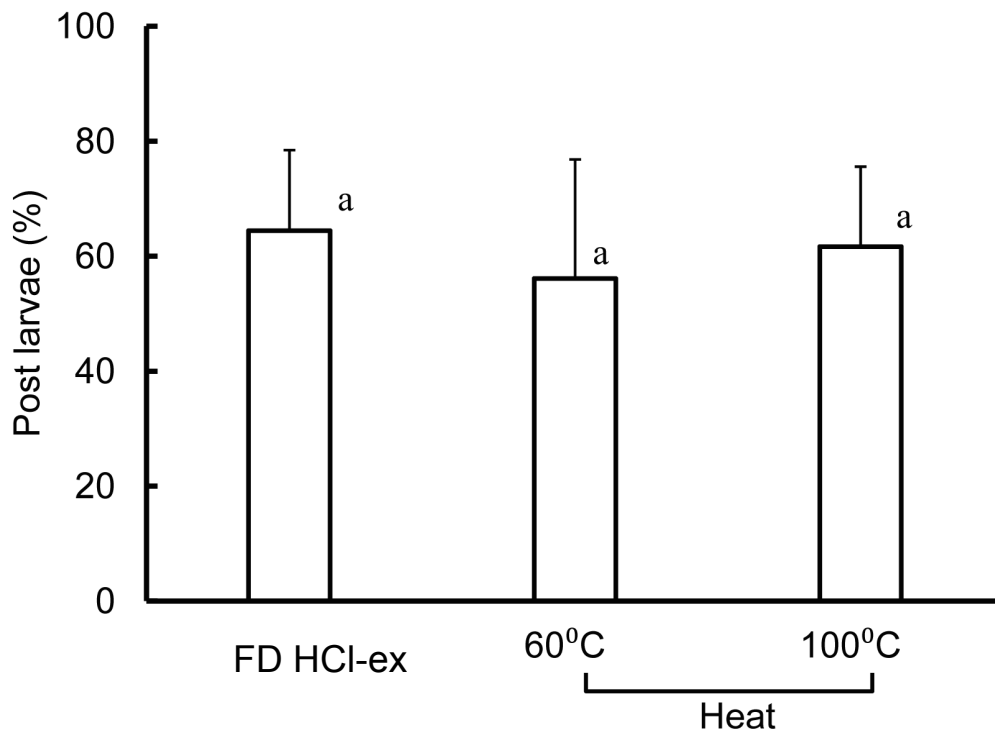


Fig. 3.1 Percentage of post larvae on *C. gigas* FD HCl-ex heated at different temperatures for 30 min.

Levels connected by the same letter are not significantly different (Tukey-Kramer HSD test, $p < 0.05$) $n=9$.

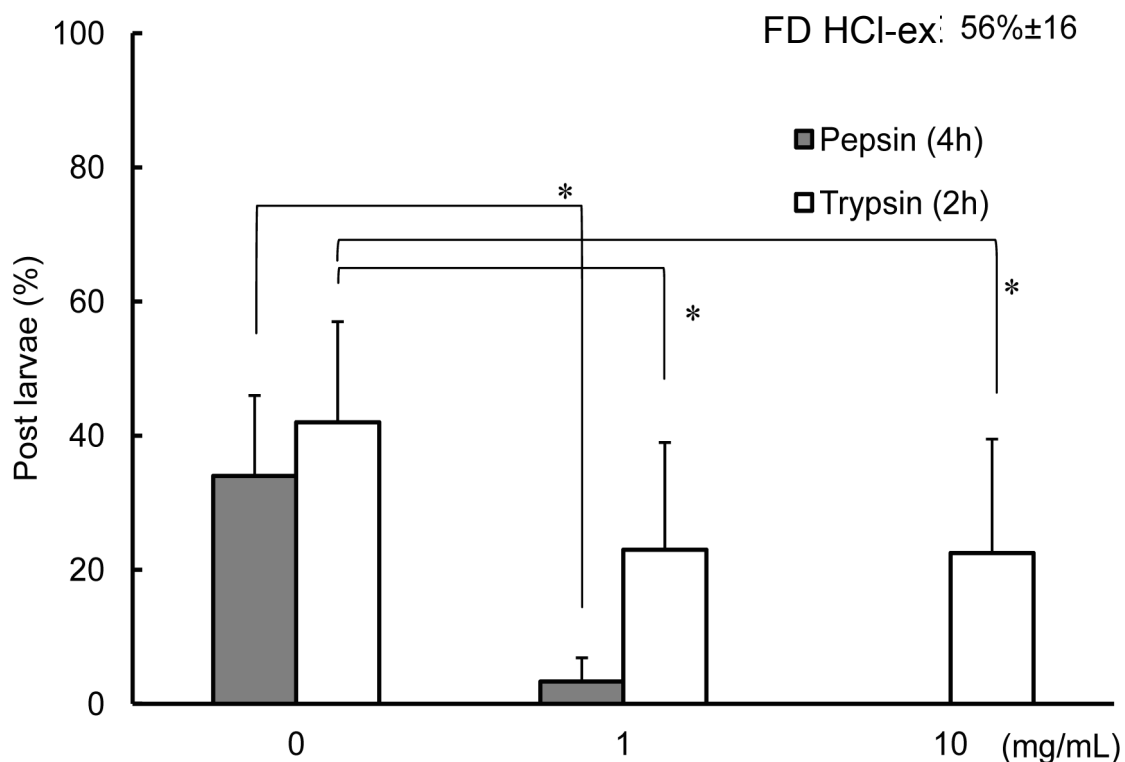


Fig. 3.2 Percentages of post larvae on pepsin and trypsin treated *C. gigas* FD HCl-ex.

Shaded and open boxes represent pepsin and trypsin treated experiments, respectively. Data are means of 9 replicates and error bars represent standard deviations (SD). Lines connected groups that were compared using Wald test. Asterisks * indicate significantly different groups ($p < 0.05$).

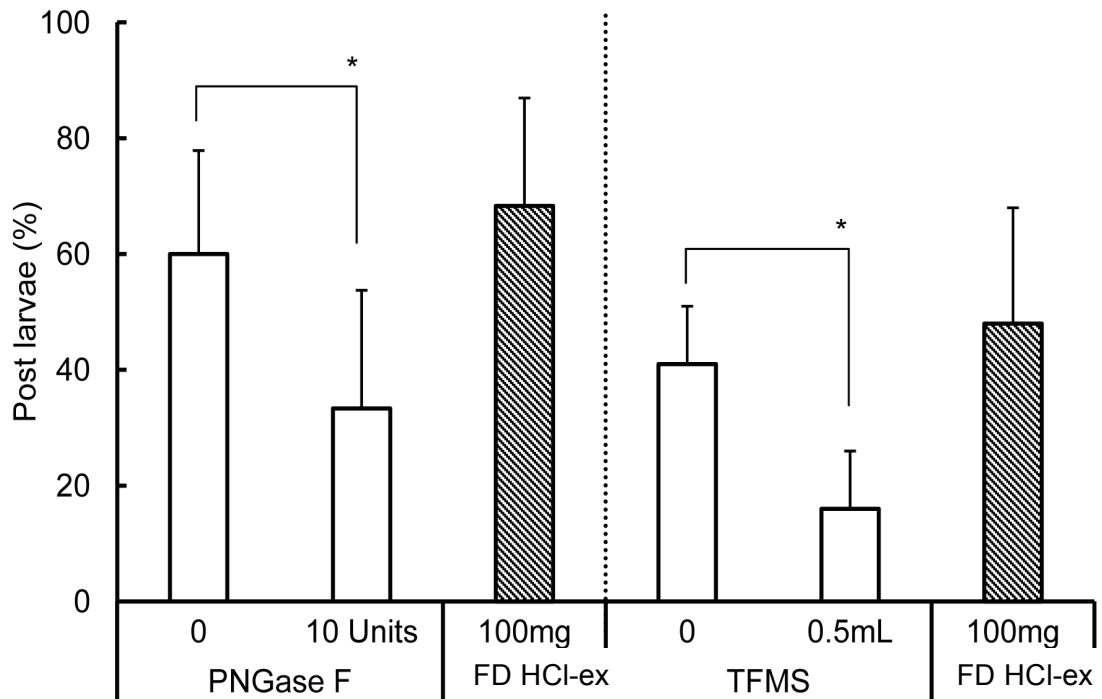


Fig. 3.3 Percentages of post larvae on PNGase F and TFMS treated *C. gigas* FD HCl-ex.

Boxes with slanting lines indicate post larvae (%) settled on FD HCl-ex 100 mg SC eq subjected to the same treatment procedure but without trypsin. Data are means of 9 replicates and error bars represent standard deviations (SD). Lines connected groups that were compared using Wald test. Asterisks * indicate significantly different groups ($p < 0.05$).

III.3.4 Effect of Lectins on the Settlement Inducing Activities of SC and FD HCl-ex

Percentages of post larvae on 50 mg SC in the presence of lentil lectin (LCA), concanavalin A (ConA), soybean lectin (SBA) and wheat germ agglutinin (WGA) at different concentrations is as shown in Fig. 3.4. In multiwell assays, larvae settled in response to 50 mg of SC (control) and the percentage of post larvae was 50% after 24 h of exposure. However, larval settlement on SC was generally affected in the presence of lectins, although the degree of effect varied with the lectin type. In both LCA and Con A at 5 and 50 $\mu\text{g mL}^{-1}$ concentrations, no larval settlement was observed but larvae were not swimming and all were observed lying at the bottom of the well with their shells closed. No mortality was also observed in these groups after 24 h. However, at 0.5 $\mu\text{g mL}^{-1}$ of LCA and Con A, percentages of post larvae were at the same level as that in the control group without lectin ($p>0.05$). Addition of SBA at 5 $\mu\text{g mL}^{-1}$ suppressed larval settlement on SC, and the percentage of post larvae was lower than the control ($p<0.05$). On the other hand, other concentrations of SBA did not affect larval settlement on SC. WGA also suppressed larval settlement on SC at all the concentrations tested, and percentages of post larvae at 0.5, 5 and 50 $\mu\text{g mL}^{-1}$ concentrations of WGA were 12%, 2% and 0%, respectively ($p<0.05$). At 5 and 50 $\mu\text{g mL}^{-1}$ of WGA, larvae exhibited similar behavior to that observed in LCA and ConA at the same concentrations. That is, larvae in these concentrations of WGA were not swimming and all were observed lying at the bottom of the well with their shells closed. No mortality was also observed in these groups after 24 h.

Percentages of post larvae on FD HCl-ex papers in the presence of LCA, ConA, SBA and WGA at different concentrations are as shown in

Fig. 3.5. In the absence of lectin (control), the percentage of post larvae on the FD HCl-ex paper was 51%, and this was at the same level as the settlement on 50 mg SC (Fig. 3.5). Larval settlement on FD HCl-ex paper in the presence of the lectins generally showed the same pattern as that on SC. That is, at 5 and 50 $\mu\text{g mL}^{-1}$ concentrations of LCA, Con A and WGA, percentages of post larvae were 0 to less than 4% and no larva was observed swimming after the 24 h assay. No mortality was also observed in these groups after 24 h. At 0.5 $\mu\text{g mL}^{-1}$ of LCA and ConA, percentages of post larvae were at the same level as that in the control group without lectin but the same concentration of WGA significantly suppressed larval settlement on the FD HCl-ex paper without affecting the swimming behavior of larvae ($p < 0.05$). Percentages of post larvae on FD HCl-ex paper in the presence of 0.5 and 5 $\mu\text{g mL}^{-1}$ SBA were significantly lower than the control ($p < 0.05$) but inhibition of settlement by SBA was not concentration dependent and larval settlement at 50 $\mu\text{g mL}^{-1}$ of SBA was the same as the control.

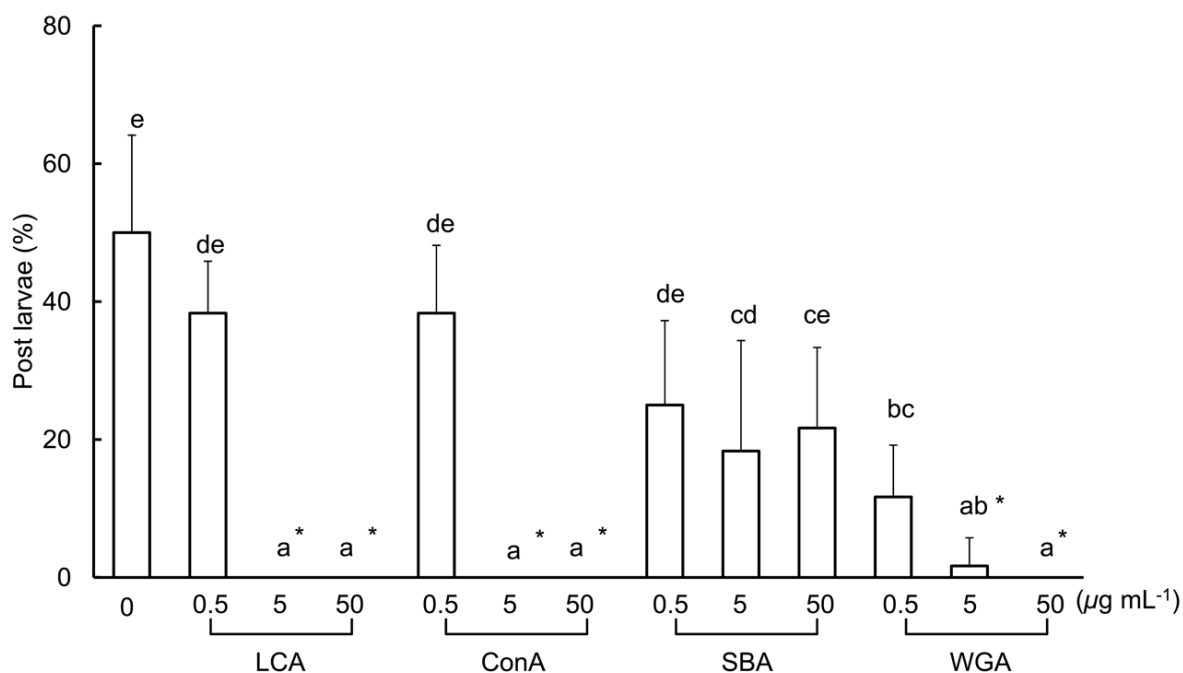


Fig. 3.4 Percentages of *C. gigas* post larvae on SC in the presence of lectins at different concentrations.

Assays were performed with 50 mg SC and FSW containing 0, 0.5, 5 and 50 $\mu\text{g mL}^{-1}$ of each lectin. Lectins were: lentil lectin (LCA), concanavalin A (ConA), soybean lectin (SBA) and wheat germ agglutinin (WGA). Data are means of 12 replicates and error bars represent standard deviations (SD). Letters indicate results of the post hoc Tukey HSD test. Groups connected by the same letter are not significantly different ($p \geq 0.05$). *Asterisks indicate lectin concentrations where larvae were not swimming.

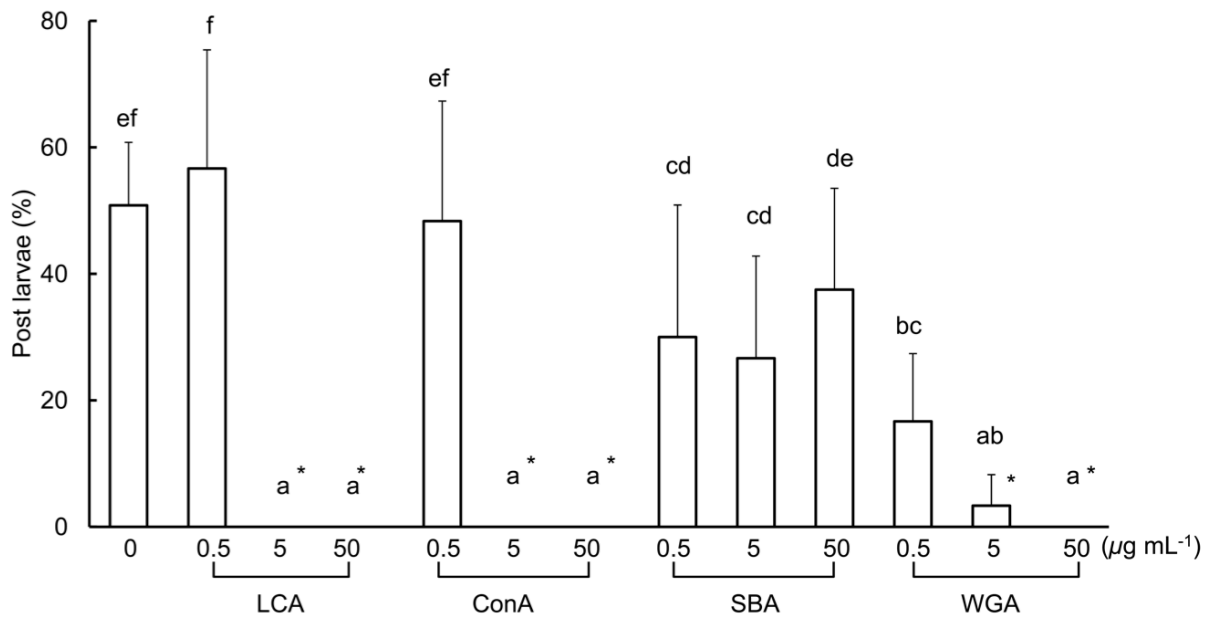


Fig. 3.5 Percentages of *C. gigas* post larvae on GF/C circle papers containing oyster shell extracts (FD HCl-ex paper) in the presence of lectins at different concentrations.

Assays were performed with GF/C filter papers containing 100 mg SC eq of FD HCl-ex (FD HCl-ex paper) and FSW containing 0, 0.5, 5 and 50 $\mu\text{g mL}^{-1}$ of each lectin. Lectins were: lentil lectin (LCA), concanavalin A (ConA), soybean lectin (SBA) and wheat germ agglutinin (WGA). Data are means of 12 replicates and error bars represent standard deviations (SD). Letters indicate results of the post hoc Tukey HSD test. Groups connected by the same letter are not significantly different ($p \geq 0.05$). *Asterisks indicate lectin concentrations where larvae were not swimming.

III.3.5 Effect of WGA on the Activity of FD HCl-ex Paper and on Pediveligers

Percentages of post larvae on FD HCl-ex papers that were treated with WGA for 2 h are as shown in Fig. 3.6A. The percentage of post larvae on the untreated FD HCl-ex paper (control) was 35%. However, treatment of the FD HCl-ex paper with WGA solutions at 0.5, 5 and 50 $\mu\text{g mL}^{-1}$ for 2 h significantly inhibited settlement in a concentration dependent manner ($p < 0.05$). Moreover, larvae that did not settle were actively swimming even after 24 h.

Percentages of post larvae on FD HCl-ex papers when pediveligers were treated with WGA at different concentrations are as shown in Fig. 3.6B. The percentage of post larvae on the FD HCl-ex paper was 48%, when pediveligers not treated with WGA were used (control). At 0.5 $\mu\text{g mL}^{-1}$ of WGA, treating pediveligers did not suppress larval settlement but the percentage of post larvae on FD HCl-ex paper significantly decreased when pediveligers treated with 5 $\mu\text{g mL}^{-1}$ of WGA were used ($p < 0.05$). By contrast, the percentage of post larvae on FD HCl-ex when pediveligers were treated with 50 $\mu\text{g mL}^{-1}$ of WGA was not significantly different to the control group, which used larvae that were not treated with WGA ($p > 0.05$). All pediveligers treated in the different concentrations of WGA solutions were actively swimming after 24 h.

III.3.6 Effects of N-Acetyl-D-glucosamine (GlcNAc) on Larval Settlement on FD HCl-ex Paper and on the Inhibiting Activity of WGA

Percentages of post larvae on FD HCl-ex papers in the presence of GlcNAc are shown in Fig. 3.7A. The percentage of post larvae on the FD HCl-ex paper (control) was 46%. However, larval settlement on the FD

HCl-ex paper significantly decreased in the presence of GlcNAc at all concentrations tested (Fig. 5A, $p < 0.05$).

Percentages of post larvae on FD HCl-ex papers when treated with $50 \mu\text{g mL}^{-1}$ of WGA and with mixtures of WGA and 10^{-6} , 10^{-5} and 10^{-4} M concentrations of GlcNAc for 2 h are shown in Fig. 3.7B. The percentage of post larvae on the FD HCl-ex paper (control) was 35%. However, larval settlement significantly decreased to 12% when the FD HCl-ex paper was treated with $50 \mu\text{g mL}^{-1}$ of WGA ($p < 0.05$). When FD HCl-ex papers were treated with mixtures of $50 \mu\text{g mL}^{-1}$ of WGA and 10^{-6} , 10^{-5} and 10^{-4} M concentrations of GlcNAc, percentages of post larvae were all at the same level as that of the control (Fig. 5B, $p > 0.05$), which was the untreated FD HCl-ex paper, indicating that GlcNAc interfered with the activity of WGA to inhibit settlement on the FD HCl-ex papers.

III.3.7 Evidence of WGA-binding Settlement Inducing Activity in SC and FD HCl-ex

Fluorescence of SC and GF/C filter papers with and without FD HCl-ex after dyeing with fluorescein isothiocyanate conjugated WGA (FITC-WGA) when viewed under UV field are shown in Fig. 3.8. Under the UV field, SC that were not dyed with FITC-WGA showed no fluorescence (Fig. 3.8B), indicating that SC had no autofluorescence. When dyed with FITC-WGA, SC showed green fluorescence (Fig. 3.8C), indicating a WGA-binding sugar present in SC. GF/C filter paper without FD HCl-ex also showed no fluorescence even after dyeing with FITC-WGA (Fig 3.10D). However, when GF/C filter papers containing $10 \mu\text{g}$ and $20 \mu\text{g}$ protein concentrations of FD HCl-ex were dyed with FITC-WGA, green fluorescence were observed in both cases (Fig. 3.8E, F), with

a brighter fluorescence in the filter paper containing 20 μg protein of FD HCl-ex (Fig. 3.8F) than the one with 10 μg protein of FD HCl-ex (Fig. 3.8E), indicating that a WGA-binding sugar was also present in the glycoprotein FD HCl-ex.

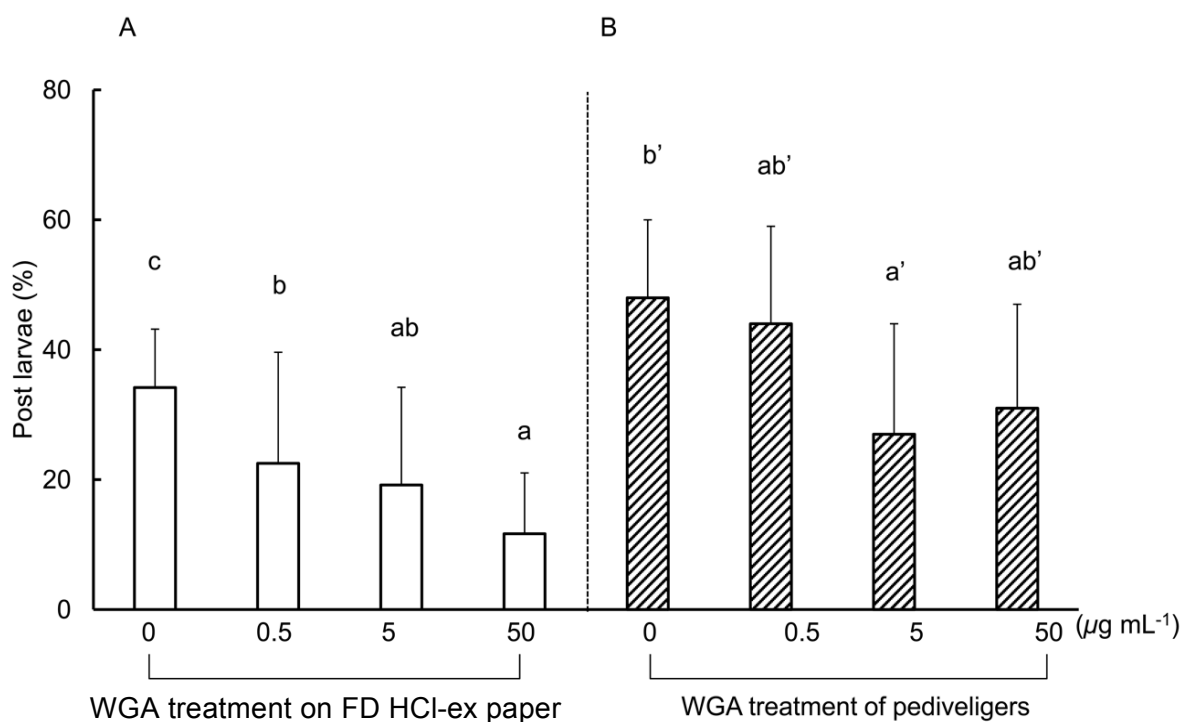


Fig. 3.6 (A, B) Percentages of *C. gigas* post larvae on FD HCl-ex paper: (A), when FD HCl-ex paper was treated with different concentrations of WGA; and (B), when pediveligers were treated with different concentrations of WGA.

GF/C filter papers containing 100 mg SC eq of FD HCl-ex (FD HCl-ex paper) were treated with WGA (0.5, 5 and 50 $\mu\text{g mL}^{-1}$) for 2 h prior to assay in (A). Pediveligers were treated with WGA (0.5, 5 and 50 $\mu\text{g mL}^{-1}$) for 2 h prior to assay with untreated FD HCl-ex paper as the substrate in (B). Data are means of 12-18 replicates and error bars represent standard deviations (SD). Small letters and those with prime marks indicate results of the post hoc Tukey HSD test. Groups connected by the same letter are not significantly different ($p \geq 0.05$).

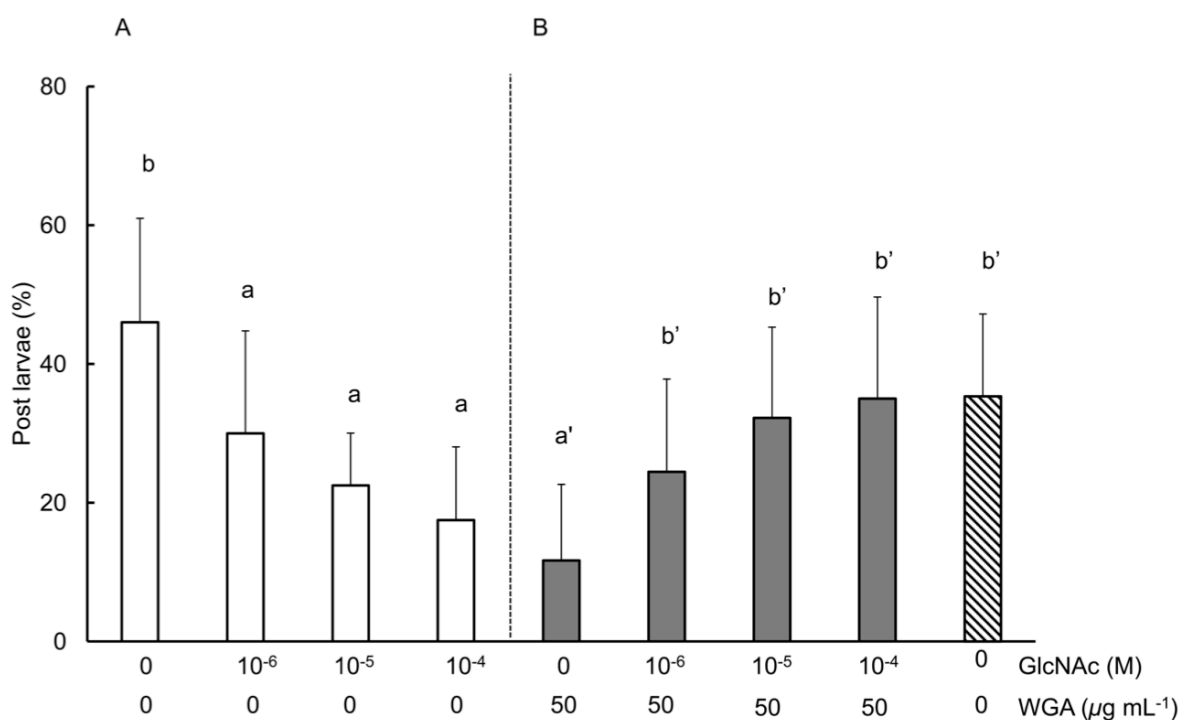


Fig. 3.7 (A, B) Percentages of *C. gigas* post larvae on FD HCl-ex papers: when in the presence of N.Acetyl-D-glucosamine (GlcNAc) (A), and when FD HCl-ex papers were treated with WGA and with mixtures of WGA and different concentrations of GlcNAc (B).

A: Blank boxes indicate percentages of post larvae on GF/C filter papers containing 100 mg SC eq of FD HCl-ex (FD HCl-ex paper) in the presence of 10^{-6} , 10^{-5} and 10^{-4} M of GlcNAc solutions. B: Shaded boxes indicate percentages of post larvae on FD HCl-ex papers treated with either WGA ($50 \mu\text{g mL}^{-1}$) only or with mixtures of WGA ($50 \mu\text{g mL}^{-1}$) and 10^{-6} to 10^{-4} M concentrations of GlcNAc for 2 h prior to assays. Box with slanting lines indicates percentage of post larvae on FD HCl-ex paper washed with FSW (control B). Data are means of 12 to 18 replicates and error bars represent standard deviations (SD). Small letters and those with prime marks indicate results of the post hoc Tukey HSD test on the treatments. Groups connected by the same letter are not significantly different ($p \geq 0.05$).

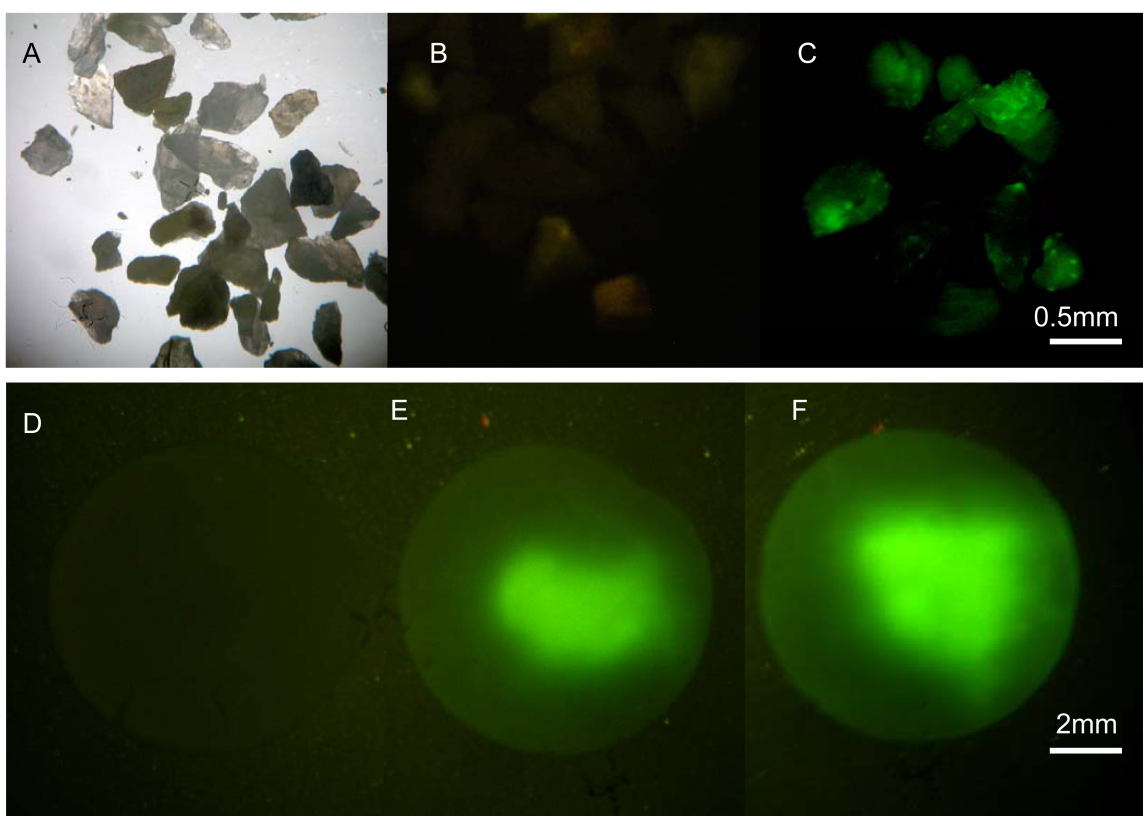


Fig. 3.8 Fluorescence of SC and GF/C filter paper containing FD HCl-ex after dyeing with fluorescein isothiocyanate (FITC) conjugated WGA.

A) SC under bright field; B) SC without FITC conjugated WGA (FITC-WGA) under UV field; C) SC after dyeing with FITC-WGA under UV field; D) FITC-WGA dyed GF/C filter paper without FD HCl-ex under UV field; E) FITC-WGA dyed GF/C filter paper containing 10 μg protein of FD HCl-ex under UV field; F) FITC-WGA dyed GF/C filter paper containing 20 μg protein of FD HCl-ex under UV field.

III.3.2 Activities of Fractions of FD HCl-ex from Gel Filtration

The distribution of FD HCl-ex following gel filtration and percentages of post larvae of the pooled fractions are as shown in Fig. 3.4.

Eluted fractions after filtration of FD HCl-ex in Superdex 200 10/300 GL column exhibited one peak with several shoulders. Eluted fractions were pooled into three, each with corresponding MW range of: 45 to 150 kDa for fraction 1; 1.3 to 44 kDa with an eluted peak at approximately 6 kDa for fraction 2; and <1.3 kDa for fraction 3 (Fig. 3.9).

Tukey HSD test showed that percentages of post larvae in response to the different concentrations of fraction 1 (F1) were all significantly higher than that of the blank control but no difference in settlement inducing activity was found between the concentrations tested. Tukey HSD test also revealed that larval settlement on all concentrations of fraction 2 (F2) and fraction 3 (F3) were the same as that of the blank control (FSW). On the other hand, no difference in the settlement inducing activities was found between 50 mg SC eq of F1 and 100 and 200 mg SC eq of F2. The percentage of post larvae in F1 at 100 mg SC eq was 55%, and this was at the same level as the activity of 100 mg SC eq of FD HCl-ex (56%) before gel filtration ($p \geq 0.05$, Tukey HSD test) (Fig. 3.9).

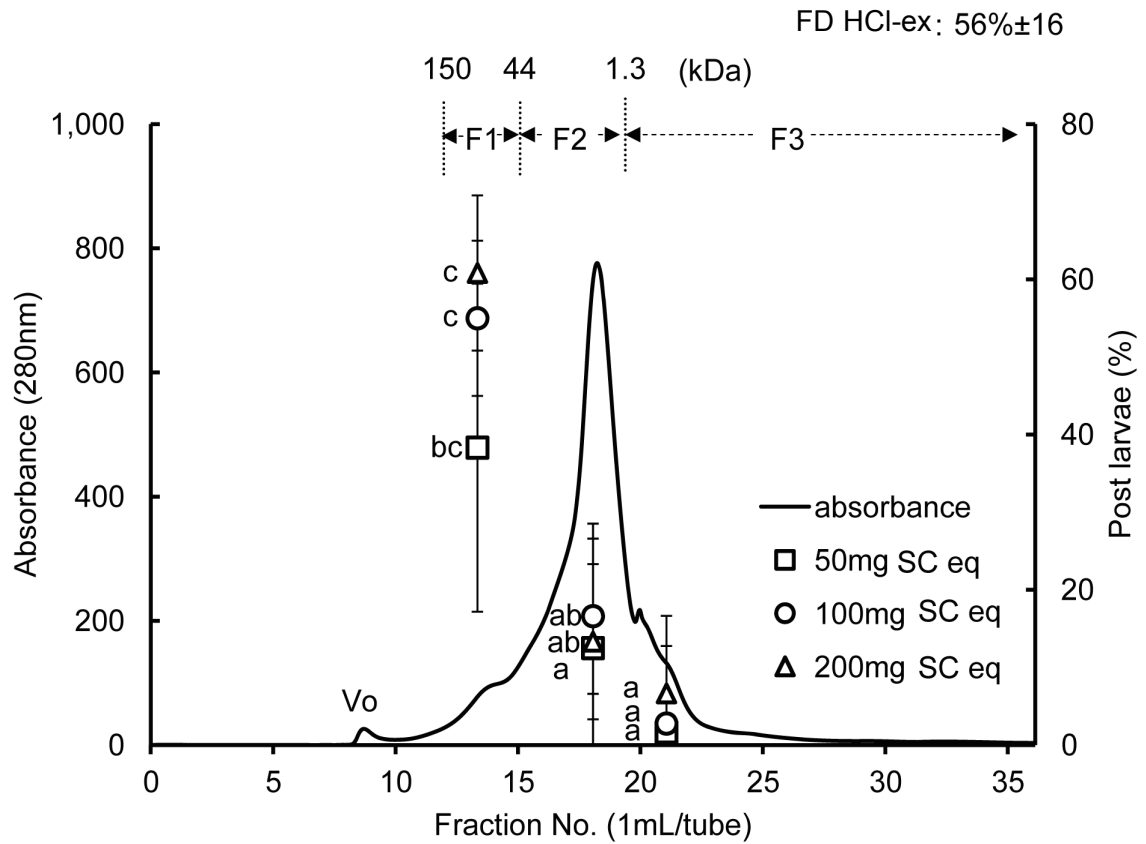


Fig. 3.9 Absorbance and eluted fractions of *C. gigas* FD HCl-ex after filtration in Superdex 200 10/300 GL column, and the percentages of post larvae at 50, 100 and 200 mg SC equivalent of each of the pooled fractions (F1: 45-150kDa, F2: 1.3-44kDa, and F3: <1.3kDa molecular mass ranges).

Vo indicates void volume. Data are means of 6 to 9 replicates and error bars represent standard deviations (SD). Letters indicate results of the post hoc Tukey HSD test. Values connected by the same letter are not significantly different ($p \geq 0.05$).

III.3.3 SDS-PAGE of FD HCl-ex and Fractions from Gel Filtration

The SDS-PAGE gel reveals the protein bands of *C. gigas* FD HCl-ex and fractions 1 and 2 (Fig. 3.10). After staining with Stains-all (Sigma-Aldrich, St. Louis MO, USA), two bands were visualized in FD HCl-ex lane; a minor band with MW of 65 kDa and a major band with 55 kDa MW. In fraction 1 lane, only one major band with MW of 55 kDa was visualized. No band was visualized in the lane of fraction 2 since the peak eluted in this fraction had a low molecular weight (approximately MW 6 kDa).

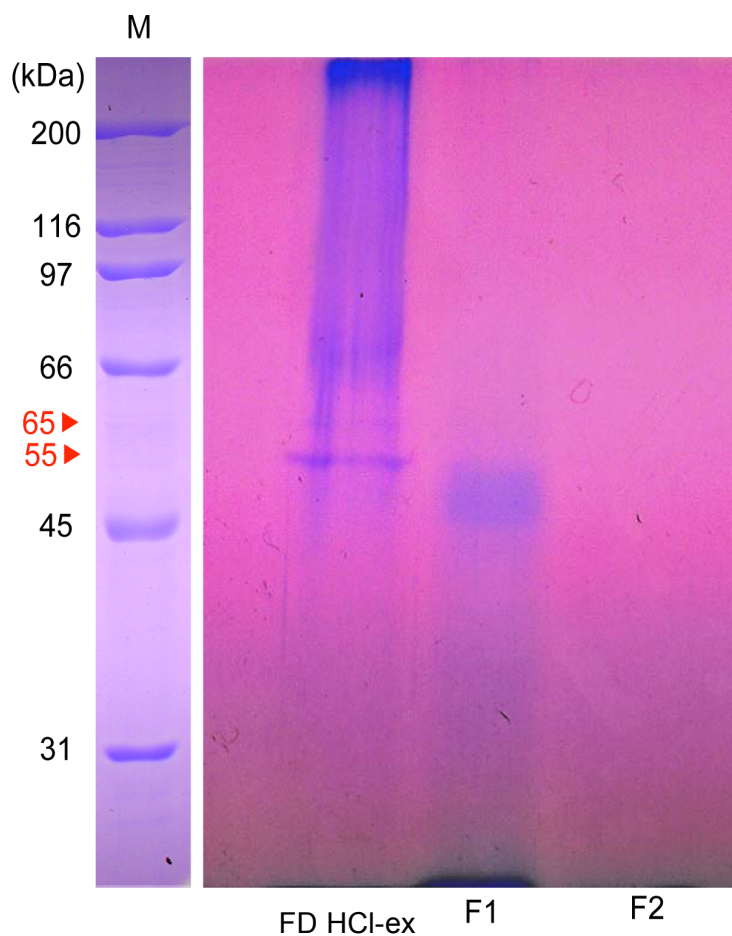


Fig. 3.10 SDS-PAGE gel image of *C. gigas* FD HCl-ex, F1 (fraction 1) and F2 (fraction 2) stained with Stains-all.

SDS-PAGE was performed on 10% polyacrylamide gels; 10 μ g protein content was loaded in each lane. M indicates the molecular weight markers. Molecular weights were estimated using the molecular weight marker “Broad Range” (BIORAD).

III.4. Discussion

It has been demonstrated that the acid-soluble matrix of *C. gigas* shells contained a settlement-inducing compound for conspecific larvae (Chapter II). Moreover, a series of treatments (heat, enzymatic and chemical) conducted on the acid-soluble settlement inducing compound suggested that the substance was a heat stable (Fig. 3.1) glycoprotein that was digested in pepsin and trypsin (Fig. 3.2), and deglycosylated with PNGase F and trifluoromethanesulfonic acid or TFMS (Fig. 3.3). Reduction in the activity of the settlement inducing compound after trypsin treatment also suggests the presence of arginine and/or lysine in the peptide because trypsin cleaves the carboxyl bond or arginine or lysine anywhere in a peptide (Olsen et al. 2004). Moreover, PNGase F cleaves between the GlcNAc and asparagine residues of *N*-linked oligosaccharides (Dwek et al. 1993).

The role of glycoproteins in the larval settlement has been described in the barnacle *A. amphitrite*, the settlement inducing compound from adult conspecifics, referred to as Settlement Inducing Protein Complex or SIPC, is also a glycoprotein of high molecular mass consisting of three major subunits of 76, 88, and 98 kDa with lentil lectin (LCA)-binding sugar chains (Matsumura et al. 1998a). The glycoprotein in the organic matrix of *C. gigas* shells that acted as settlement cue was acid-soluble and insoluble in water, as demonstrated in the control groups of the treatment experiments (Fig. 3.2 and Fig. 3.3) where the settlement cue was absorbed and remained intact in GF/C substrates even after washing with FSW. This insoluble settlement cue in *C. gigas* shells may account for the high recruitment of *C. gigas* on shells of dead conspecifics that was observed by

Diederich (2005) in the Oosterschelde estuary (Netherlands), and may be a chemical basis of larval settlement on conspecifics.

The insoluble nature of this cue suggests that the cue is recognized by *C. gigas* larva only after direct contact with the substrate. Hence, the swimming oyster larva may encounter the cue on a substrate (e.g. shells of conspecifics) through the mediation of other cues from the environment. Water soluble cues may play a role in the commencement of the settlement/searching behavior of the planktonic oyster larvae. Waterborne cues from bacteria (Fitt et al. 1990; Tamburri et al. 1992) and conspecifics (Tamburri et al. 1992; Hirata et al. 2008) have been reported to mediate the settlement behavior of oyster larvae. Tamburri et al. (1992) reported that *C. virginica* larvae responded similarly to waterborne substances released both from adult oysters and from biofilms, that is, swimming in a manner indicative of settlement behavior. Coon et al. (1990b) demonstrated that *C. gigas* larvae exhibited settlement behavior that typically precedes cementation and metamorphosis when exposed to ammonia and suggested that ammonia may be a natural environmental cue. Fitt and Coon (1992) reported that adult oysters produce enough NH_3 needed to induce larval settlement behavior, and that concentrations of NH_3 in the water near oyster shells in oyster beds is enough to trigger this behavior. Thus, Bonar et al. (1990) suggested that if a larva enters an area of elevated NH_3 , as for example in a dense community assemblage such as an oyster bed, it begins the stereotyped searching behavior, and if the environment is acceptable, the additional cues the larva receives (tactile, physical, chemical, etc.) promote ultimate metamorphosis. Bao et al. (2007b) also suggested the involvement of two chemical cues, a waterborne and a surface bound cue, in the larval settlement of *M. galloprovincialis*. Further investigation may

clarify possible synergistic influence of both waterborne and surface bound cues on larval settlement of *C. gigas*.

Glycoproteins have been implicated as inducers of larval settlement in some marine invertebrates (Maki and Mitchell, 1985; Matsumura et al., 1998b). In such models, lectin receptors on the surface of larvae are thought to recognize specific sugar compounds of the settlement inducer during settlement (Kirchman et al., 1982b). In the present investigation, we have demonstrated that lectins affected larval settlement on SC and FD HCl-ex (Fig. 3.4 and Fig. 3.5), both of which contained the presumed settlement inducer of *C. gigas* larvae. However, at high concentration levels of LCA, ConA and WGA, larvae were observed to have ceased swimming, indicating that the effect of lectins at such concentrations was more on larval behavior than on the presumed settlement inducer. With larvae closing their shells and just lying at the bottom of the well, it can be assumed that larvae were not able to proceed to searching behavior and contact the settlement inducer in SC and FD HCl-ex. This may be more sensible than the alternative explanation that lectins may have covered specific sugar chains of the presumed settlement inducer in SC and FD HCl-ex. By contrast, lower concentrations of SBA ($<5 \mu\text{g mL}^{-1}$) and WGA ($0.5 \mu\text{g mL}^{-1}$) affected larval settlement on SC and FD HCl-ex without affecting the swimming behavior of the larvae during the assay, even though SBA seems to be less effective than WGA. SBA and WGA have different sugar specificity; the former preferentially binding to N-acetylgalactosamine and galactose while the latter, to N-acetylglucosamine (reviewed by Lis and Sharon, 1986). This difference in sugar specificity may account for the difference in effectiveness of the two lectins, although this warrants further investigation. Moreover, results with SBA were

inconsistent regardless of the concentration used, and therefore only WGA was further pursued in this study.

Whether WGA acts on the presumed settlement inducer in FD HCl-ex or to the larva itself was also investigated. Treating GF/C filter paper containing FD HCl-ex with different concentrations of WGA resulted in WGA effectively inhibiting larval settlement on FD HCl-ex in a manner dependent on the concentration of WGA used (Fig. 3.6A). WGA may have acted directly on the FD HCl-ex by binding to the carbohydrate moiety of the presumed settlement inducer in FD HCl-ex. This method of blocking sugar compounds on a surface with the settlement inducer using commercial lectins to check the role of sugar in larval settlement has been used by other researchers (Kirchman et al., 1982b; Matsumura et al., 1998a; Jouuchi et al., 2007). Matsumura et al. (1998a) and Jouuchi et al. (2007), in separate experiments, effectively inhibited cyprid larval settlement on surfaces with the adult extract of *A. amphitrite* and with the diatom film by treating the surfaces with LCA, and concluded that LCA binding sugar compounds in *A. amphitrite* extract and on periphytic diatoms are directly involved in the larval settlement of *A. amphitrite*. Treating *C. gigas* pediveligers that were used in the assay with $5 \mu\text{g mL}^{-1}$ of WGA also reduced settlement on GF/C filter paper containing FD HCl-ex (Fig. 3.6B). WGA may have bound to shell matrix glycoproteins in the internal organs and muco-polysaccharides on the foot of the oyster larvae (Cranfield 1973; Johnstone et al. 2008), and thus somehow interfered with the *C. gigas* larval settlement behavior. Another explanation could be that WGA may have no direct effect on oyster larvae at all; treating larvae at a higher concentration ($50 \mu\text{g mL}^{-1}$) of WGA did not significantly reduce

settlement. Matsumura et al. (1998a) also treated *A. amphitrite* cyprids with LCA, but LCA had no direct effect on their larvae.

Kirchman et al. (1982b) demonstrated that in the polychaete, *J. brasiliensis*, both glucose and ConA blocked the settlement and metamorphosis inductive effect of bacterial films, and postulated that settlement and metamorphosis of *J. brasiliensis* is induced by the binding of larval lectins to extracellular polysaccharides or glycoproteins produced by bacterial films. Although the species studied and origins of the settlement inducers are different, similarities of our findings to that reported by Kirchman et al. (1982b) highlights the possibility that conspecific shell glycoprotein-induced settlement of *C. gigas* larvae is also mediated by oyster lectin-like receptors. In some ascidians, the receptor for settlement cues from bacterial films is a mannose-binding lectin (MBL), which activates metamorphosis via an MBL-complement pathway of innate immunity (Davidson and Swalla 2002; Woods et al. 2004; Roberts et al. 2007). In bivalves, lectins are present in the mucus covering pallial organs (gills, labial palps) of the oyster *C. virginica* (Espinosa et al. 2009; Jing et al. 2011) and the mussel *Mytilus edulis* (Espinosa et al. 2010a; Espinosa et al. 2010b), and these are suspected to be involved in particle capture and sorting.

Low molecular weight sugars can inhibit lectin-mediated processes, since the added sugar competes for the carbohydrate-binding site on the protein (reviewed by Lis and Sharon, 1986). In the present investigation, GlcNAc dissolved in FSW inhibited settlement of *C. gigas* larvae on FD HCl-ex paper (Fig. 3.7A) suggesting that the sugar may have bound to a larval lectin that has specificity for GlcNAc. Kirchman et al. (1982b) also

reported that glucose inhibited larval settlement of *J. brasiliensis* on bacterial films. Moreover, GlcNAc, the sugar to which WGA preferentially binds to (Nagata and Burger, 1974), interfered with the suppression of settlement by WGA (Fig. 3.7B). This observation suggests that a WGA-binding sugar may be contained in the settlement inducer in FD HCl-ex. In the barnacle *A. amphitrite*, lentil lectin (LCA) inhibited adult extract-induced settlement but the addition of α -D-mannopyranoside, which is known to bind to LCA, cancelled the suppression of settlement by LCA (Matsumura et al., 1998a). Chitin has been found among the chemical components of the shells of *C. gigas* (Choi and Kim 2000; Lee and Choi 2007) and chitin is a crystalline polymer of 1,4 linked N-acetyl-D-glucosamine (reviewed by Merzendorfer and Zimoch, 2003). Fluorescence microscopy observation of dyed SC and FD HCl-ex on GF/C filter papers confirmed the binding of WGA to a sugar chain compound (Fig. 3.8).

Finally, gel filtration of the matrix compound of *C. gigas* shells resulted in the settlement cue being eluted between 45 and 150 kDa (Fig. 3.9). SDS-PAGE also confirmed the settlement inducing compound to have a major protein band with a molecular weight of 55 kDa (Fig. 3.10). The band was visualized after staining with Stains-all, which is used to dye phosphoproteins (Green et al. 1973; Goldberg and Warner 1997). Stains-all will also stain in blue those proteins that may have cation-binding potential (Campbell et al. 1983).

In conclusion, the settlement cue was a heat stable 55 kDa glycoprotein component in the shell organic matrix. Moreover, *C. gigas* larval settlement on the glycoprotein from shells of conspecifics involves a specific lectin-sugar interaction. The N-Acetyl-D-glucosamine containing

carbohydrate moiety in the shell matrix glycoprotein may play a direct role in the induction of settlement of *C. gigas* larvae by conspecific shells.

IV. General Discussion and Conclusions

In the search for settlement inducing compounds for *C. gigas* larvae, researchers have focused on biofilms and conspecifics (Hirata 1998; Hirata 2005; Tamburri et al. 2007) as sources of cues. In the present study, the author focused on larval settlement behavior in response to shells of conspecifics. Firstly, the author checked whether *C. gigas* oyster larvae would respond differently to different mollusks shells and then extracted the settlement cue from conspecific shells (Chapter II). After extraction, the settlement cue was then characterized and partially purified (Chapter III).

In chapter II, results clearly indicated that *C. gigas* larvae settled in response to shells of its conspecifics or closely related species. This finding suggests that larvae may recognize shells of its own or related species during settlement and will respond favorably by settling in high numbers. This gregarious settlement triggered by conspecific shells in *C. gigas* has been observed in the field and has been interpreted as the difference in the texture of shells and that larvae exhibited preference for rough oyster shells over mussels smooth shells (Diederich 2005).

The high settlement response of *C. gigas* larvae to shells of its own species and *C. nippona* suggests that larvae may recognize a settlement cue that may be abundant in conspecific shells and closely related species. *C. gigas* shells contain more than 99% calcium carbonate and 0.5%, by weight, of some organic matrices (Marie et al. 2011). These organic matrices, which consist of polysaccharides and proteins, show great diversity in their molecular weights and amino acid compositions among molluscan species, and their secretion is fundamentally controlled by genes involved in shell formation (Kobayashi and Samata 2006). The differences in matrix

components of the shells among species may also be another explanation for the lower settlement response of *C. gigas* larvae to other species.

Molluscan shell matrix proteins have shown stability at high temperatures (Totten et al. 1972; Akiyama 1978). This characteristic may explain the result where the putative settlement cue in shells of *C. gigas* was stable even at 200°C and decreased in activity only when it was charred at 300°C. Similarly, Knight-Jones (1953) heated pieces of slates covered with bases of newly detached barnacles and observed that the activity of the settlement cue from *Balanus balanoides* shells remained unaffected until 200°C but this was gradually destroyed at about 250°C. The fact that *C. gigas* SC retained settlement inducing activity even after heat and antibiotic treatments –normally used to eliminate biofilms or bacterial constituents (Lau and Qian 2001; Bao et al. 2007a)– suggests that the settlement cue was contained in *C. gigas* shell itself and distinct from the cue in bacterial biofilms reported in early studies (Fitt et al. 1989).

Aqueous and organic solvent extracts of *C. gigas* shells did not significantly induce larval settlement at all concentrations tested, indicating that these solvents did not efficiently extract the chemical cue. By contrast, the HCl extract of *C. gigas* shells showed settlement inducing activity that was equivalent to the activity of the shell itself. Therefore, HCl proved to be the most efficient solvent to use in extracting the settlement-inducing compound in *C. gigas* shells. Acids are often used in the demineralization of shells to collect the protein matrix (Totten et al. 1972; Akiyama 1978; Mann et al. 2012). Results indicate that the acid-soluble matrix of *C. gigas* shells contained a settlement-inducing compound for conspecific larvae.

In the study of identifying settlement-inducing compounds of different marine benthic invertebrates, it is important to select a suitable substrate that is inert and is not repelled by the larva being studied, and at the same time is easy to handle or manipulate. The author tested larval settlement behavior of *C. gigas* on plaster plates, nitrocellulose membrane, GF/C filter papers, and glass. We found that all substrates tested were inert and, as is, did not induce larval settlement. On the other hand, adding the FD HCl-ex to these substrates resulted in larval settlement on all substrates, although larvae settled differentially on the different substrates, settling in greatest numbers on GF/C filter paper and glass. However, GF/C filter papers are easier to handle in the assays. Thus, GF/C filter paper was chosen as the substrate for *C. gigas* larval settlement assays in this study.

In chapter III, treatments conducted on the oyster shell extract when applied on GF/C, suggested that the substance was a heat stable glycoprotein that was digested in pepsin and trypsin, and deglycosylated with PNGase F and trifluoromethanesulfonic acid or TFMS. Reduction in the activity of the settlement inducing compound after trypsin treatment also suggests the presence of arginine and/or lysine in the peptide because trypsin cleaves the carboxyl bond or arginine or lysine anywhere in a peptide (Olsen et al. 2004). Moreover, PNGase F cleaves between the GlcNAc and asparagine residues of *N*-linked oligosaccharides (Dwek et al. 1993).

Glycoproteins have been implicated as inducers of larval settlement in some marine invertebrates (Maki and Mitchell, 1985; Matsumura et al., 1998b). In those cases, lectin receptors on the surface of larvae are thought to recognize specific sugar compounds of the settlement inducer during

settlement (Kirchman et al., 1982b). Thus, in Chapter III, the author also demonstrated that some lectins tested at low concentrations (WGA [$0.5 \mu\text{g mL}^{-1}$]; SBA [$<5 \mu\text{g mL}^{-1}$]) affected larval settlement on SC and on the acid extract prepared from conspecific shells, both of which contained the presumed settlement inducer of *C. gigas* larvae without affecting the swimming behavior of the larvae during the assay. SBA and WGA have different sugar specificity; the former preferentially binding to N-acetylgalactosamine and galactose while the latter, to N-acetylglucosamine (reviewed by Lis and Sharon, 1986). This difference in sugar specificity may account for the difference in effectiveness of the two lectins, although this warrants further investigation. Moreover, results with SBA were inconsistent regardless of the concentration used, and therefore only WGA was further pursued in this study. Because WGA acts on the presumed settlement inducer in FD HCl-ex and not on to the larva itself, evidence suggested that WGA may have bind to the carbohydrate moiety of the presumed settlement inducer in FD HCl-ex. Matsumura et al. (1998a) and Jouuchi et al. (2007), in separate experiments, effectively inhibited cyprid larval settlement on surfaces with the adult extract of *A. amphitrite* and with the diatom film by treating the surfaces with LCA, and concluded that LCA binding sugar compounds in *A. amphitrite* extract and on periphytic diatoms are directly involved in the larval settlement of *A. amphitrite*.

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Low molecular weight sugars can inhibit lectin-mediated processes, since the added sugar competes for the carbohydrate-binding site on the protein (reviewed by Lis and Sharon, 1986). In the present investigation, GlcNAc inhibited settlement of *C. gigas* larvae on FD HCl-ex paper suggesting that the sugar may have bound to a larval lectin that has specificity for GlcNAc. Kirchman et al. (1982b) also reported that glucose inhibited larval settlement of *J. brasiliensis* on bacterial films. Moreover, GlcNAc, the sugar to which WGA preferentially binds to (Nagata and Burger, 1974), interfered with the suppression of settlement by WGA. Similar results have been reported for *A. amphitrite*, when lentil lectin (LCA) inhibited adult extract-induced settlement but the addition of α -D-mannopyranoside, which is known to bind to LCA, cancelled the suppression of settlement by LCA (Matsumura et al., 1998a). Results suggest that a WGA-binding sugar may be contained in the settlement inducer in FD HCl-ex. This was confirmed with fluorescence microscopy observation of dyed SC and FD HCl-ex on GF/C filter papers. Chitin has been found among the chemical components of the shells of *C. gigas* (Choi and Kim 2000; Lee and Choi 2007) and chitin is a crystalline polymer of 1,4 linked N-acetyl-D-glucosamine (reviewed by Merzendorfer and Zimoch, 2003).

The purification of the acidic proteins from mineralized tissues is technically problematic as explained by Gotliv et al. (2003). As these

macromolecules are intimately associated with the mineral phase and are highly charged (Cariolou and Morse 1988), they are very difficult to extract from the mineralized tissue and once extracted the molecules readily aggregate and are thus difficult to separate by chromatography (Gotliv et al. 2003). Furthermore, when the assemblages of acidic macromolecules from mineralized tissues are subjected to separation by gel electrophoresis, some of the observed bands are diffuse and smeared, whereas others are sharply, poorly or unstained by Coomassie Blue and silver staining (Dauphin and Cuif 1997; Gotliv et al. 2003). Despite these technical problems, gel filtration of the FD HCl-ex resulted in the settlement cue being eluted between 45 and 150 kDa. SDS-PAGE also confirmed the settlement inducing compound to have a major protein band with a molecular weight of 55 kDa. The band was visualized after staining with Stains-all, which is used to dye phosphoproteins (Green et al. 1973; Goldberg and Warner 1997).

Therefore, the author suggests a 2-step model for larval settlement of *C. gigas* on conspecifics (Fig. 4.1). Firstly, competent oyster larva may encounter the cue on a substrate (e.g. shells of conspecifics) through the mediation of waterborne cues from the environment such as NH_3 from bacteria (Tamburri et al. 1992; Fitt et al. 1990; Fitt and Coon 1992) and conspecifics (Tamburri et al. 1992; Hirata et al. 2008) (Fig. 4.1-①). Fitt and Coon (1992) reported that adult oysters produce enough NH_3 needed to induce larval settlement behavior. Bonar et al. (1990) suggested that if a larva enters an area of elevated NH_3 , as for example in a dense community assemblage such as an oyster bed, it begins the stereotyped searching behavior. Secondly, when the larva of *C. gigas* commences its searching behavior, it will recognize/respond to a WGA binding glycoprotein on the

substrate, resulting in the settlement, cementation and metamorphosis. This glycoprotein is usually abundant in the shells of conspecifics than in shells of other molluscan species (Fig. 4.1-②).

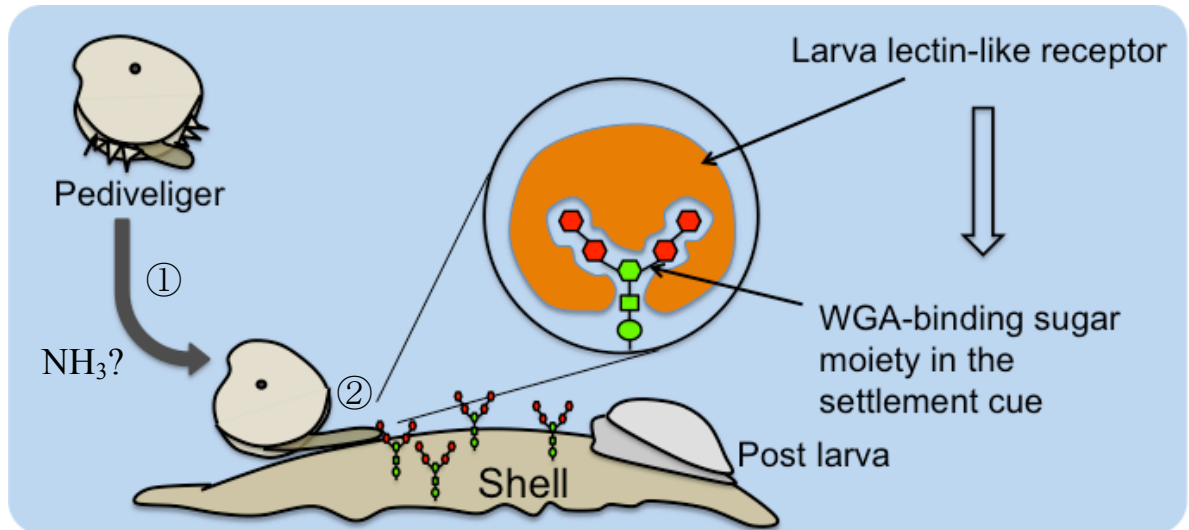


Fig. 4.1 Model of *C. gigas* larval settlement on conspecific shells.

In conclusion *C. gigas* larvae settled in higher numbers on shells of its conspecifics and *C. nippona*, in response to a heat stable glycoprotein component in the organic matrix and its settlement mechanism involves a sugar and a WGA lectin-like receptor interaction. This is the first report of an insoluble glycoprotein in the organic matrix of *C. gigas* shells that acts as a chemical cue for larval settlement of conspecifics. Moreover, The findings here can also have application in the aquaculture of this commercially important species in regions where natural seed is not available.

V. References

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