RESEARCH ARTICLE

Structural characterisation of the *N*-glycan moiety of the barnacle settlement-inducing protein complex (SIPC)

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SUMMARY

Many barnacle species are gregarious and their cypris larvae display a remarkable ability to explore surfaces before committing to permanent attachment. The chemical cue to gregarious settlement behaviour – the settlement-inducing protein complex (SIPC) – is an α_2-macroglobulin-like glycoprotein. This cuticular protein may also be involved in cyprid reversible adhesion if its presence is confirmed in footprints of adhesive deposited during exploratory behaviour, which increase the attractiveness of surfaces and signal other cyprids to settle. The full-length open-reading frame of the SIPC gene encodes a protein of 1547 amino acids with seven potential *N*-glycosylation sites. In this study on *Balanus amphitrite*, glycan profiling of the SIPC *via* hydrophilic interaction liquid chromatography with fluorescence detection (HILIC-fluorescence) provided evidence of predominantly high mannose glycans (M2–9), with the occurrence of monofucosylated oligomannose glycans (F(6)M2–4) in lower proportions. The high mannose glycosylation found supports previous observations of an interaction with mannose-binding lectins and exogenous mannose increasing settlement in *B. amphitrite* cypris larvae. Transmission electron microscopy of the deglycosylated SIPC revealed a multi-lobed globular protein with a diameter of ~8 nm. Obtaining a complete structural characterisation of the SIPC remains a goal that has the potential to inspire solutions to the age-old problem of barnacle fouling.

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Key words: *Balanus amphitrite*, biofouling, glycosylation, mannose, pheromone.

INTRODUCTION

The settlement-inducing protein complex (SIPC) is a contact pheromone that has been identified as the primary mediator of gregarious settlement in the barnacle *Balanus amphitrite*, though other chemical signals are involved (Clare, 2011). Barnacles are a key component of marine biofouling – the unwanted accumulation of biological material on man-made submerged surfaces. There are strong economic and environmental drivers to better understand the mechanisms involved in fouling and to devise effective antifouling technologies.

The barnacle life cycle involves sessile adult and mobile nauplius and cypris larval stages. The adults of most species studied cross-fertilise by pseudocopulation, requiring a compatible mate to be within reach of the penis. Gregarious settlement behaviour is an adaptive strategy to facilitate successful mating. The SIPC, which is implicated in both adult–larva and larva–larva interactions, is expressed throughout the barnacle life cycle (Matsumura et al., 1998c) and has been localised to the cuticle (Dreanno et al., 2006a).

The SIPC of *B. amphitrite* has a deglycosylated mass of ~170 kDa (predicted from the amino acid sequence) and an apparent native molecular mass of ~260 kDa (estimated from SDS-PAGE comparison with standards). The SIPC contains three major subunits of 76, 88 and 98 kDa (Matsumura et al., 1998b). These polypeptide chains play an important role in the settlement of *B. amphitrite* larvae

(Matsumura et al., 1998b). Each subunit, when assayed individually, induced cyprid settlement as effectively as the intact SIPC (Matsumura et al., 1998c). The gene sequence of the SIPC encodes a 1547 amino acid protein with seven potential *N*-glycosylation sites. There is significant homology to the highly *N*-glycosylated α2-macroglobulin (A2M) and insect thioester-containing proteins (TEP) (Dreanno et al., 2006). Cyprid temporary adhesive also appears to be glycoprotein based (Clare and Matsumura, 2000). Therefore, glycoproteins potentially function as both settlement cues and bioadhesives in barnacles.

Glycans on the cell surface play an important role in recognition both on cell surfaces and in solution (Ambrosi et al., 2005). There are other instances of surface-associated proteins playing similar roles to the SIPC in other marine organisms. The harpacticoid copepod *Tigriopus japonicus* secretes a protein with sequence similarities to A2M that has been shown to assist in mate recognition, mate guarding and spermatophore transfer through chemical contact (Ting and Snell, 2003). Similarly, a glycoprotein is involved in mate recognition in calanoid copepods (Snell and Carmona, 1994). Glycoproteins may also function as attractants for allospecifics; for example, eggs of the horseshoe crab, *Limulus polyphemus*, are used as bait in eel fisheries and it is thought that egg-derived glycoproteins act as chemo-attractants (Ferrari and Targett, 2003). In some marine organisms the settlement cue must come from live animals, such

as for the European flat oyster, *Ostrea edulis* (Crisp, 1965). There is also evidence for a settlement cue associated with oyster shell biofilm as bacterial films removed from oyster shells also caused changes in settlement activity (Tamburri et al., 1992). For barnacles, the cue appears to effect a settling response from living, dead or preserved animals (Crisp, 1965). Newly moulted or recently settled individuals strongly induce settlement activity (Crisp and Meadows, 1962), and it has been observed that even old cement bases on surfaces will attract cyprids (Knight-Jones, 1953).

To add to the understanding of glycans and their role in settlement, it is also important to understand the structure of the core protein of the SIPC. The general properties of barnacle settlement factors have been studied intensely for many years, focusing on bioassays and the reaction of proteins to varying physical conditions (Larman et al., 1982). Indeed, Yamamoto synthesised polypeptide models of *Balanus balanoides* (=*Semibalanus balanoides*) cement in the hope of developing compatible bioadhesives (Yamamoto, 1992). Here, we present a characterisation of the glycosylation pattern of the SIPC protein and initial structural characterisations using transmission electron microscopy (TEM).

An understanding of the mechanisms behind the gregarious settlement of organisms such as barnacles may highlight ways to interfere with unwanted fouling by these animals, e.g. through the development of antagonists. In an effort to further understand the contribution of the glycan moieties to the biological activity of the SIPC, the present study reports on the characterisation of the *N*-linked glycans present on this glycoprotein, in particular the potential presence of high mannose-type glycans, as mannose has previously been shown to affect settlement (Matsumura et al., 1998a).

MATERIALS AND METHODS
Purification of the SIPC

The striped acorn barnacle *Balanus amphitrite* (=*Amphibalanus amphitrite*) Darwin (Clare and Høeg, 2008) from North Carolina was maintained in an aquarium where individual broods of barnacles were kept in separate plastic tanks containing natural filtered (0.45 μm) seawater at 26°C, on a 12 h:12 h light:dark cycle. Tanks were aerated and the water changed daily. The adult broodstock were fed newly hatched *Artemia* sp. (Artemia International, Fairview, TX, USA) nauplii daily. Adults were cleaned thoroughly with water, briefly dried and frozen for extraction of the SIPC. The barnacles were roughly crushed for 30 min using a pestle and mortar; 150% volume of 50 mmol l^{-1} Tris-HCl pH 7.5 was added during further crushing. The protein mixture was stirred for 2 h, then filtered through 200 μm paper to remove larger particles. The filtrate was centrifuged at 13,000 *g* for 30 min, retaining the supernatant, which was further filtered through glass fibre filter paper (Whatman No. 3, Whatman, Maidstone, Kent, UK). For every litre of filtered supernatant, 472 g of ammonium sulphate $(NH_4)_2SO_4$ was added slowly, and this was stirred overnight before centrifuging at 13,000 *g* for 30 min, retaining the pellet. The pellet was re-suspended in a small volume of 50 mmol l^{-1} Tris-HCl pH 7.5, transferred to dialysis tubing (12,000 M_w cut-off) and dialysed in 3 l of 50 mmol l^{-1} Tris-HCl pH 7.5 overnight, changing the buffer after 3 then 6 h. The content of the dialysis tubing was centrifuged for 3 h at 13,000 *g*, and the supernatant filtered using 0.2 μm cellulose acetate filter (Whatman) under vacuum. The filtrate constituted the total protein from the barnacles and total protein assays were carried out. The protein was diluted to 1:10, 1:50 and 1:100, and was compared with six dilutions from 0 to 1 mg ml^{-1} of BSA (Sigma, Poole, Dorset, UK) at the same dye concentration (Total Protein Reagent, BioRad,

Hemel Hempstead, Herts, UK). The total protein obtained was purified by ion exchange chromatography for elution by charge using an EconoPump system and EconoSystem fraction collector (Bio-Rad). A 15 mm diameter, 100 cm column containing Q-Sepharose cation exchanger (Pharmacia Biotech, GE Healthcare, Little Chalfont, Bucks, UK) was equilibrated with 1 l of 50 mmol l^{-1} Tris-HCl pH 7.5; 100 mg of crude extract was diluted in 30 ml of 50 mmol l^{-1} Tris-HCl pH 7.5. Gradient buffers of 50 mmol l^{-1} Tris-HCl pH 7.5 and 1 mol l^{-1} NaCl were used to run a gradient at 1 ml min^{-1}. Fractions of 3 ml were collected on ice. Total protein assays were carried out on every second fraction of the unknown sample. The SIPC was eluted between 100 and 120 min when detected by SDS-PAGE with confirmatory immunoblotting using anti-rabbit IgG (Sigma) as previously outlined (Cutler, 2004). Following this, gel filtration for elution by size using the same system and the following setup was carried out. A 15 mm diameter, 100 cm long column containing Sephacryl S-200 size exclusion media (Pharmacia Biotech) was used with buffers consisting of a mixture of 2 mol l^{-1} NaCl, 1.5 mol l^{-1} Tris-HCl pH 7.5 and dH$_2$O. The column was run at 0.5 ml min^{-1}, collecting fractions of 3 ml on ice. Again, the SIPC was eluted at 25–37 min when detected by SDS-PAGE with confirmatory immunoblotting (Matsumura et al., 1998c).

N-glycan release and labelling

N-glycans were directly released from one-dimensional (1-D) SDS-PAGE gel bands or gel blocks as previously described (Royle et al., 2006). Briefly, the gel bands were excised and washed repeatedly with successive washes of 100% acetonitrile and 20 mmol l^{-1} sodium bicarbonate buffer pH 7.0 while shaking on an orbital platform shaker (Heidolph, Schwabach, Germany) for 15 min. This ensured maximum recovery of glycans. After washing, glycans were liberated enzymatically with PNGase F (Prozyme, San Leandro, CA, USA) and 20 mmol l^{-1} sodium bicarbonate buffer (pH 7.0) at 37°C overnight. PNGaseF cleaves between the GlcNAc and asparagine residues of *N*-linked oligosaccharides. It does not cleave *O*-linked, *C*-linked or *N*-linked glycans containing α-1–3 core fucose. The released glycans were extracted from the gel pieces after digestion by successive washing and sonicating for 15 min at 37°C with water and finally with 100% acetonitrile. All extractions were combined and concentrated *via* vacuum centrifugation.

Once dry, the *N*-glycans were treated with 20 μl of 1% v/v formic acid for 40 min to convert the released glycosylamines back to reducing sugars before redrying. Glycans for HPLC analysis were then labelled with 5 μl of 2-aminobenzamide (2-AB) using the LudgerTag^{TM} 2-AB kit (Ludger, Abingdon, Oxon, UK), vortexed for 10 min and incubated for 30 min at 65°C. Excess 2-AB was removed using solid-phase extraction by Whatman 3MM chromatography paper; 5 μl of the 2-AB-labelled sample was applied to the paper and allowed to dry, and 100% acetonitrile was used as the mobile phase. Labelled glycans were eluted by syringing dH$_2$O through the paper, in 4×500 μl aliquots of dH$_2$O, leaving the water in contact with the paper for 10 min in between water changes. All water was retained, combined and dried for analysis by HPLC (Royle et al., 2006).

HILIC-fluorescence glycan profiling

Dried samples were re-suspended with 20 μl dH$_2$O and 80 μl 100% acetonitrile. HILIC-fluorescence was performed using a Waters Alliance 2695 Separations Module with a Waters 2475 Multi Wavelength Fluorescence Detector (Waters Corporation, Millford, MA, USA). The detection wavelengths used were λ_{ex}=330 nm and

λ_{em}=420 nm. Separations were performed on a TSKgel Amide-80 5 µm (250×4.6 mm) column. A 180 min gradient of 50 mmol l^{-1} ammonium formate pH 4.4 and 100% acetonitrile was used for glycan separation as previously described (Royle et al., 2006) (20% ammonium formate:80% acetonitrile to 80% ammonium formate:20% acetonitrile over 180 min). The system was calibrated by running an external standard of 2-AB-labelled dextran ladder (5 µl dextran, 15 µl dH$_2$O and 80 µl acetonitrile) (2-AB–glucose homopolymer, Ludger), which was used for annotation of the experimental data with glucose unit (GU) values using Empower GPC software. Throughout this research, carbohydrate structures and names are as per the nomenclature system outlined elsewhere (Harvey et al., 2009).

Structural assignments and exoglycosidase digestion
Aliquots of the labelled glycans were digested with the following exoglycosidase enzymes: bovine kidney α-fucosidase (BKF) and Jack-bean α-mannosidase (JBM; Prozyme) in 1 µl of 50 mmol l^{-1} sodium acetate buffer pH 5.5, the required enzyme and dH$_2$O to make up to 10 µl, at 37°C for 16 h. Digested glycans were separated from enzyme by centrifugation through 10 kDa M_w cut-off enzyme filters (Millipore, Billerica, MA, USA) and then analysed by HILIC-fluorescence as outlined above. Structural assignments were made based on enzyme activity and incremental shifts in GU.

Sugars in solution
Following characterisation of the glycans on the SIPC, experiments were carried out using sugars in solution to investigate the efficacy of the dominant sugar, mannose, as a cue when in solution. One day old *B. amphitrite* cyprids were exposed to different concentrations of two sugars (methyl-α-mannopyranoside and methyl-β-galactopyranoside), 3-isobutyl-1-methylxanthine (IBMX) and the native SIPC for 24 h. An artificial seawater (ASW) control was also included. Settlement of 10 cyprids per well in a 24-well plate (Iwaki Cell Biology, Iwaki, Japan) was measured as the percentage of cyprids settled or metamorphosed compared with the total number present after 24 h. The following dilutions were used to produce 6 replicates of each dilution: for mannose, galactose and IBMX: 1 mmol l^{-1}, 0.1 mmol l^{-1}, 0.01 mmol l^{-1}, 1 µmol l^{-1} and 0.1 µmol l^{-1}; and for the SIPC: 50 nmol l^{-1}, 25 nmol l^{-1}, 5 nmol l^{-1}, 2.5 nmol l^{-1} and 0.5 nmol l^{-1}. Because of the potential for the sugars to degrade during the freeze–thaw processes, these solutions were made up fresh for each experiment. Minitab probit analysis on the mannose and galactose data uses the concentrations and number settled to give a half-maximal effective concentration (EC$_{50}$) estimate for each replicate; these were then averaged to give the mean EC$_{50}$ for each treatment. Galactose was chosen as a control as it has been used in comparative sugar experiments previously (Khandeparker et al., 2002). Although the glycans in the SIPC are

N-linked, when there is more than one glycan present, in the case of M2 to M9, they are *O*-linked to each other at the anomeric position. Consequently, using *O*-linked glycans was expected to produce a similar settlement effect.

Transmission electron microscopy
Native and deglycosylated samples of the SIPC were diluted to ~0.7 and 0.07 mg ml^{-1} with 50 mmol l^{-1} Tris-HCl pH 7.5. Samples were prepared for TEM by the 'single-droplet' parafilm procedure (Harris and Horne, 1991). Briefly, a copper grid (400-mesh) coated with carbon film was surface activated by glow discharge (Edwards Coating Unit) before the 10 µl sample was loaded, then washed and stained with 2% uranyl acetate at pH 4.2–4.5. This heavy metal-containing cation forms a layer of negative stain, whereby the proteins are surrounded by the stain, which scatters electrons revealing the protein as white particles on a darker background. TEM was performed on a Philips CM100 transmission electron microscope with CCD camera, and digital images of areas of interest were taken at 130,000× instrumental magnification. Protein concentration and sample conditions were optimised to generate grids with a homogeneous coverage of single particles before image processing. The glycosylated form of the SIPC protein aggregated under the conditions used for imaging; therefore, the protein was deglycosylated to produce optimal loading on EM grids for imaging. Deglycosylated SIPC in a 10% solution was chosen as the optimal dilution. Two-dimensional (2-D) image processing provided preliminary image averaging to give a qualitative assessment of the protein structure. 2-D processing involved selection of individual protein particles that were processed by multivariate analysis to determine the shape of the protein and define any surface projections.

To produce 2-D class averages, digital micrograph images were processed in e2boxer to select individual particles of interest and then EMAN2 (National Center for Macromolecular Imaging, http://ncmi.bcm.edu/ncmi) to extract the boxed particles and stack them. As particles lay at random orientations in the micrographs, to remove noise and create class averages, multivariate analysis was run on the 266 individual protein particle images, again using EMAN2, as described elsewhere (Tang et al., 2007).

RESULTS
HILIC-fluorescence profiling of enzymatically released *N*-glycans from the SIPC
N-Glycans released *via* overnight incubation with PNGaseF (*N*-glycosidase F) were labelled with 2-aminobenzamide and profiled using HILIC-fluorescence on an amide stationary phase as previously described (Royle et al., 2006). The resulting chromatographic data presented in Fig. 1 were annotated with GU values by comparison with a dextran hydroslate ladder. Initial structural assignment of the glycans present in the chromatographic
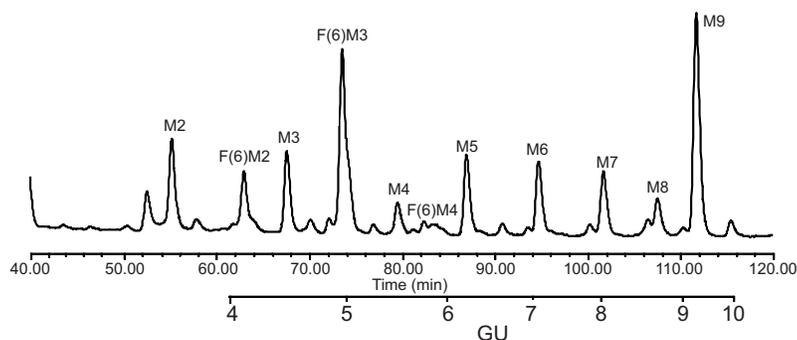

Fig. 1. Hydrophilic interaction liquid chromatography (HILIC)-fluorescence chromatogram of the glycans released from *Balanus amphitrite* native settlement-inducing protein complex (SIPC) in a gel block showing the oligomannose series M2–9 and the core fucosylated derivatives F(6)M2–4. GU, glucose units.

Table 1. Structure details of the glycans of *B. amphitrite* SIPC with glucose unit (GU) and percentage area from the HILIC-fluorescence data

Glycan	HPLC GU	% Area[a]
M2	3.57	6.7
F(6)M2	4.08	4.9
M3	4.42	5.2
F(6)M3	4.89	15
M4	5.43	2.1
F(6)M4	5.7	0.7
M5	6.19	5.3
M6D3	7.11	5.1
M7 or M7D1	8.06	4.4
M8	8.93	2.8
M9	9.61	15.5

[a]The amount of glycan present as a percentage of the total glycans measured by HPLC.
HILIC, hydrophilic interaction liquid chromatography.

peaks was performed by comparison of the experimental GU data with GlycoBase (Campbell et al., 2008). Table 1 lists the initial assignments for the glycans present in each peak, along with the relative proportions of each glycan present. From Table 1 the

experimental data suggest that high mannose-type glycans are the most prevalent glycans present in the native SIPC, forming a mannose ladder consisting of the oligomannose series M2–9. Monofucosylated oligomannose species [F(6)M2–4] were also found to be present, albeit in considerably lower quantities.

Exoglycosidase digestions of the *N*-glycan pool liberated from the SIPC were also performed in an attempt to refine the preliminary glycan structural assignments. The resulting HILIC-fluorescence traces of the *N*-glycan pool after exoglycosidase digestion are depicted in Fig. 2. Fig. 2A shows the oligomannose series M2–9 and core fucosylated oligomannose glycans F(6)M2–4 released from the native SIPC, while Fig. 2B shows the JBM digest of the glycans, wherein all peaks present were found to be trimmed back, yielding only M1 and F(6)M1. Fig. 2C is the resulting HILIC-fluorescence chromatogram following digestion with BKF, which shows the removal of the α1–6-linked fucose residue of the reducing terminal *N*-acetylglucosamine (GlcNAc) residue in F(6)M2–4 to yield M2, M3 and M4, respectively. In addition, digestion of the F(6)M4 peak revealed a previously masked peak corresponding to a monoantennary glycan (A1). All other peaks associated with the oligomannose series M2–M9 were unaffected by the presence of α-fucosidase (BKF), indicating the absence of an α1–6-linked fucose residue on their reducing terminal GlcNAc residue. Fig. 2D shows the gel blank.
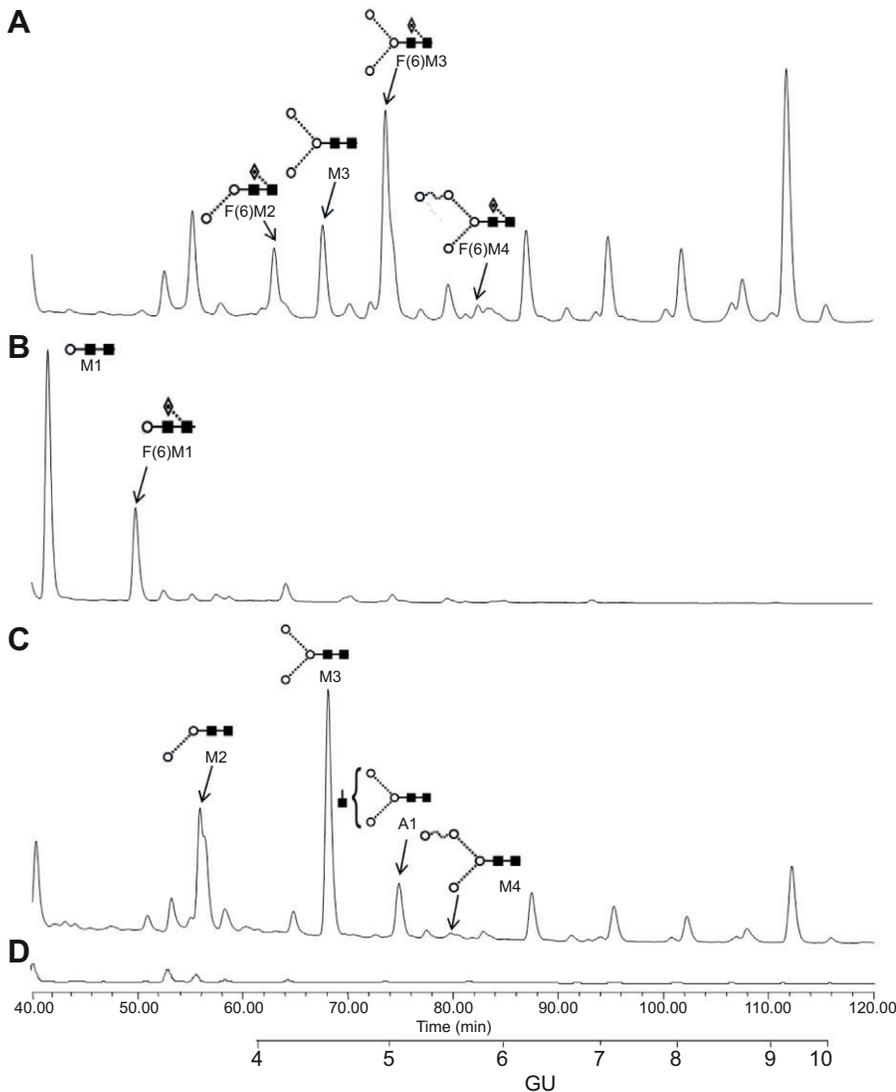

Fig. 2. (A) HILIC-fluorescence chromatogram of the glycans released from the native SIPC in a gel block showing the oligomannose series M2–9 with F(6)M2–4. (B) Chromatogram showing the Jack-bean α-mannosidase (JBM) digest of the glycans. (C) Chromatogram showing the bovine kidney α-fucosidase (BKF) digest of the glycans. (D) Chromatogram showing the gel blank. Molecular representations of the sugars are included. The individual monosaccharides are represented as described previously (Harvey et al., 2009).

Sugars in solution

The response of settling cyprids to different concentrations of sugars and other controls is shown in Fig. 3. Fig. 3A indicates that cyprids settle at a higher rate when exposed to exogenous mannose than to galactose. Cyprid settlement in $1\,\mathrm{mmol\,l^{-1}}$ mannose did not differ from that in the ASW control but was significantly different compared with settlement in $1\,\mathrm{mmol^{-1}}$, $1\,\mathrm{\mu mol^{-1}}$ and $0.1\,\mathrm{\mu mol^{-1}}$ galactose (data were not normal, Kolmogorov–Smirnov test statistic=0.121, $P<0.01$, equally variant; Levene's test statistic=0.37, $P=0.320$, significantly different; Kruskall–Wallis H_{10}=26.37, $P=0.003$). The SIPC elicited a similar maximum response to mannose (Fig. 3B); however, the concentrations involved were very different. To achieve around 40% settlement, $1\,\mathrm{mmol\,l^{-1}}$ of mannose was required, whereas only $25\,\mathrm{nmol\,l^{-1}}$ of the SIPC achieved this response. It is noticeable in Fig. 3 that in solutions that cue settlement (mannose and the SIPC), there was a decline in settlement with decreasing concentration. However, in the non-cueing case of galactose, settlement remained approximately constant at all concentrations. Fig. 3C is the exception to this, and as IBMX is known to be a strong inducer of cyprid settlement, the much higher levels of settlement were expected. However, it appears that at the highest concentrations, IBMX stops all settlement. These results corroborate previous findings (Clare et al., 1995) that concentrations of more than $0.1\,\mathrm{mmol\,l^{-1}}$ elicit very little or zero settlement. This was followed by a peak in settlement of around 70% at $0.01\,\mathrm{mmol\,l^{-1}}$ then a gradual decline with decreasing concentration to $0.1\,\mathrm{\mu mol\,l^{-1}}$.

From the settlement data, the EC_{50} for settlement was determined using Minitab probit analysis. After $24\,\mathrm{h}$, galactose EC_{50} was $2.29\pm1\,\mathrm{mmol\,l^{-1}}$, mannose EC_{50} was $1.548\pm0.23\,\mathrm{mmol\,l^{-1}}$ and the SIPC EC_{50} was $102\pm40.1\,\mathrm{nmol\,l^{-1}}$.

Transmission electron microscopy

Deglycosylated SIPC appeared homogeneous and globular by TEM imaging of negatively stained particles. 2-D class averages of the protein were calculated using multivariate statistical analysis. This revealed a multi-lobed view of the globular protein with a diameter of ~8 nm; no obvious symmetry was present in the images. The nine class averages produced (representing slightly different molecular orientations) are shown in Fig. 4B. Because of the protein adopting a limited number of preferred orientations on the TEM grids under the sample conditions used and the limitations in the quantity of the SIPC available for analysis, it was not possible to carry out three-dimensional (3-D) reconstruction of the protein or attempt crystallisation. However, some attempts can be made to draw structural comparisons from sequence similarity searches of protein databanks carried out by Dreanno et al. (Dreanno et al., 2006). These identified proteins similar to the SIPC belonging to the α2-macroglobulin family, and included complement-like factors such as the TEP1r protein (PDBid 2PN5) from the anti-parasite immune system of the mosquito *Anopheles gambiae* (Baxter et al., 2007). The amino acid sequence for the SIPC (supplementary material Fig. S1) along with ESPript sequence alignment (Gouet et al., 1999) for TEP1r and the SIPC (supplementary material Fig. S2) are shown in the supplementary data. The two sequences appear analogous and display 26% homology. However, the main difference is that the SIPC does not contain the GCGEQ signature sequence of the thioester bond. Fig. 5 shows a visualisation overlay of the mature SIPC protein (deglycosylated) onto the full structure of mature TEP1r macroglobulin, showing structural similarities. This structure potentially illustrates how the protein might display glycans for detection by cyprids.
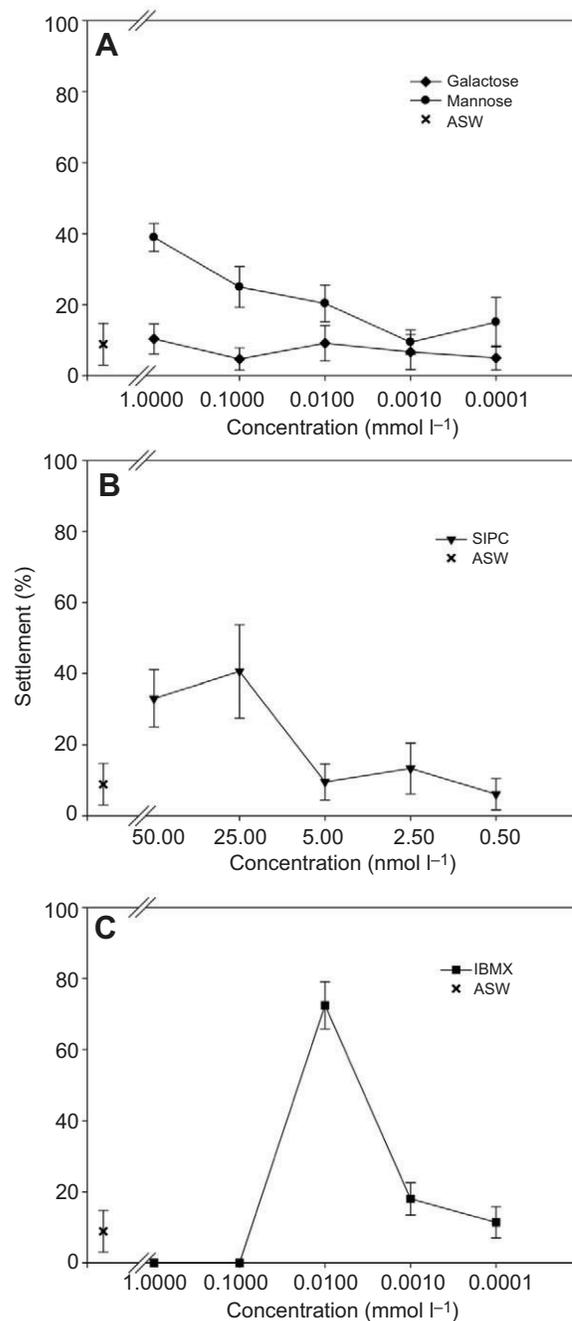

Fig. 3. Effect of exogenous sugars on the settlement of 1 day old *B. amphitrite* cyprids when compared with artificial sea water (ASW). (A) mannose and galactose at 24 h. (B) The SIPC at 24 h. (C) IBMX at 24 h. Note the different scales on the *x*-axes.

DISCUSSION

Cell surface oligosaccharides play an important role in recognition both on cell surfaces and in solution (Ambrosi et al., 2005; Caldwell and Pagett, 2010). The presence of the glycoprotein SIPC in barnacle cuticle is a key factor in the gregarious settlement of barnacles. The aim of this study was to characterise the glycosylation present on the SIPC to help reveal the role of the glycans in barnacle settlement. Through understanding the process, further progress can be made in reducing settlement. *N*-Linked glycans enzymatically liberated from the SIPC were found to be high mannose-type,
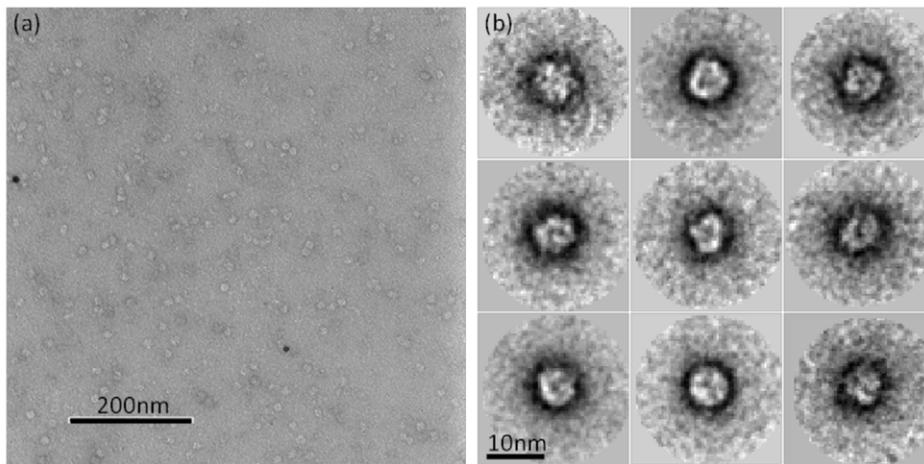
Fig. 4 (A) A representative transmission electron microscopy (TEM) image of deglycosylated *B. amphitrite* SIPC (0.07 mg ml⁻¹) using 2% w/v uranyl acetate stain on a carbon film. (B) The nine class averages, based on 266 protein particles of the deglycosylated SIPC.

ranging from M2 to M9. Previous studies have reported the presence of similar oligosaccharide structures on the proteins isolated from other aquatic organisms such as haemocyanin from the crayfish *Astacus leptodactylus* (Tseneklidou-Stoeter et al., 1995) and haemoglobins from the hydrothermal vent tube worm *Riftia pachyptila* (Zal et al., 1998).

The high mannose-type oligosaccharide structures identified on the SIPC expressed by *B. amphitrite* are in agreement with previous studies in which lectin inhibition experiments were performed. Both lentil lectin (LCA) and jack-bean lectin (ConA) were reported to inhibit settlement of *B. amphitrite*, with both lectins displaying affinity for mannose. The glucosamine- and galactose-binding lectins, wheat germ agglutinin (WGA) and peanut agglutinin (PNA), respectively, do not inhibit settlement (Matsumura et al., 1998a). Mannose in solution has also been shown to act as a settlement cue whereas galactose and glucose do not (Khandeparker et al., 2002). Dreanno and colleagues identified seven potential *N*-glycosylation sites in the amino acid sequence of the SIPC (Dreanno et al., 2006). Future work could use other exoglycosidases such as PNGaseA, which releases *N*-linked glycans with an α1–3- or α1–6-linked fucose residue on the reducing terminal GlcNAc. PNGaseA digestion is performed at the glycopeptide level, which limits further protein structural analysis. For this reason alone, it was not investigated in the current study.

Moreover, the present study found that the EC_{50} for mannose was less than that for galactose. The large standard errors seen with these EC_{50} values are common in bioassays where there is positive feedback from newly metamorphosed barnacles providing additional settlement cues to exploring cyprids. The SIPC induces consistently high settlement as it is the barnacles' natural settlement pheromone. However, this settlement is only approximately half of the maximal response to IBMX. This may be due to the SIPC forming the part of the cascade to initiate settlement, whereas IBMX may act further downstream, possibly by-passing this cascade and initiating settlement directly; a theory proposed previously (Clare et al., 1995). The efficacy of the SIPC was reduced with decreasing concentration. The pattern of results is very similar to those found by Matsumura and colleagues in both settled and metamorphosed cyprids (Matsumura et al., 1998a). Similarly, the same result was observed by Dreanno and colleagues investigating the settlement response of *B. amphitrite* cyprids to the SIPC but using the SIPC glycoprotein bound to nitrocellulose membrane (Dreanno et al., 2007).

Cyprid settlement in response to the control monosaccharide, exogenous galactose (Kruskall–Wallis H_{10}=26.37, P=0.003, where

only mannose was significantly different), was not significantly different to the ASW control at all concentrations tested. These assay results, taken together with the glycan analysis data, suggest that the mannose oligosaccharides of the SIPC contribute to the settlement-inducing activity of the glycoprotein pheromone.

The study of deglycosylated SIPC by TEM, taken in conjunction with existing protein sequence data (Dreanno et al., 2006) showing similarities to α_2-macroglobulin-like proteins, represents the first steps to understanding the protein structure of *B. amphitrite* SIPC and relating structure to function. Ting and Snell similarly discussed a contact protein from the copepod *Tigriopus japonicas*, which also possesses a likeness to the α_2-macroglobulin family (Ting and Snell, 2003). This contact protein has been implicated in mate recognition, similar to barnacles using the SIPC to identify conspecifics. A recently published conceptual model of copepod mate recognition may provide some insight into the mechanism of action of the SIPC. Snell proposed that male antennules contain receptors to female mate-recognition proteins (Snell, 2011). These proteins are secreted through ducts on the female exoskeleton and displayed but not tightly
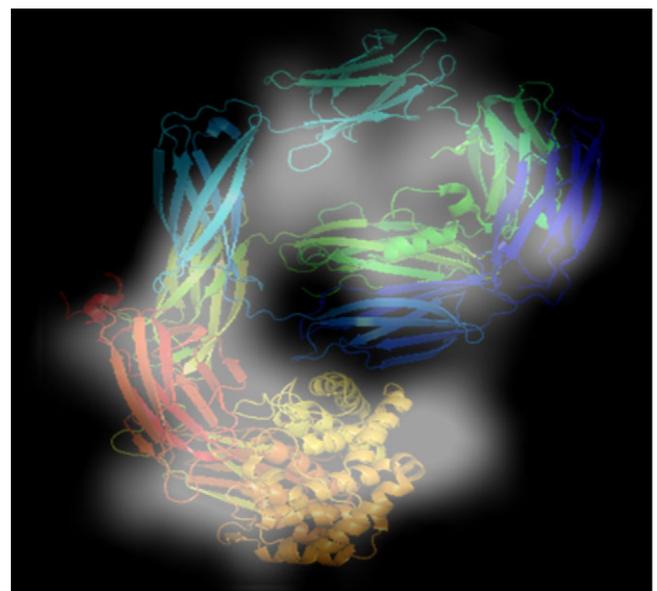

Fig. 5 Visual overlay of the structure of TEP1r (colour) and one of the SIPC class averages (white), indicating structural similarity.

bound to the surface. The proteins are thought to activate a response in the male though a protease-trapping mechanism, although the exact interaction is not yet known and it is not even clear that a 'lock and key' receptor–ligand interaction is required for signal detection (Franco et al., 2011). Although the TEM results presented in this paper are only initial analyses of the protein, this research presents the first view of the SIPC structure and provides a valuable starting point for increasing understanding of how the SIPC functions. The similarity of the SIPC to other proteins with a complement-like structure hints at the role of the protein in self/non-self recognition as the complement system functions as a defence mechanism in invertebrates, although this requires the presence of the thioester bond (Kawabata and Tsuda, 2002).

There is a considerable body of work on the protein structure of other aquatic organisms, using similar TEM protocols to that used here (Harris and Horne, 1991), mainly focusing on haemoglobins and haemocyanins. Studies on the branchiopod crustacean *Triops cancriformis* showed detailed information on the structure and function of the haemoglobin. TEM images revealed both circular shapes of 14 nm diameter and rectangular structures of 16×9 nm (Rousselot et al., 2006). Again focusing on haemocyanin, Meissner and colleagues presented the structure for the haemocyanin of the European spiny lobster *Palinurus elephas*, using a combination of cryoelectron microscopy and analysis of the amino acid sequence (Meissner et al., 2003). Comparison of the sequence with that of the American counterpart, *Panulirus interruptus*, showed more than 80% sequence homology.

The results gained through this research have provided some insight into the *N*-glycan moiety of *B. amphitrite* SIPC, indicating that mannose is present at high levels. The settlement assays suggest that mannose, as a terminal monosaccharide, may contribute to the settlement of cyprids. However, the true signal is likely to be a complex oligosaccharide. It is hoped that more advanced glycan analysis and additional assays using the deglycosylated protein would establish further examination of the SIPC glycans within the scope of this research. As cryoelectron microscopy and crystal structure determination of the SIPC to create a full 3-D molecular model remains elusive, the current knowledge will be built upon further with continued characterisation of the SIPC protein.

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