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Barnacle cement: a polymerization model based on evolutionary concepts

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SUMMARY

Enzymes and biochemical mechanisms essential to survival are under extreme selective pressure and are highly conserved through evolutionary time. We applied this evolutionary concept to barnacle cement polymerization, a process critical to barnacle fitness that involves aggregation and cross-linking of proteins. The biochemical mechanisms of cement polymerization remain largely unknown. We hypothesized that this process is biochemically similar to blood clotting, a critical physiological response that is also based on aggregation and cross-linking of proteins. Like key elements of vertebrate and invertebrate blood clotting, barnacle cement polymerization was shown to involve proteolytic activation of enzymes and structural precursors, transglutaminase cross-linking and assembly of fibrous proteins. Proteolytic activation of structural proteins maximizes the potential for bonding interactions with other proteins and with the surface. Transglutaminase cross-linking reinforces cement integrity. Remarkably, epitopes and sequences homologous to bovine trypsin and human transglutaminase were identified in barnacle cement with tandem mass spectrometry and/or western blotting. Akin to blood clotting, the peptides generated during proteolytic activation functioned as signal molecules, linking a molecular level event (protein aggregation) to a behavioral response (barnacle larval settlement). Our results draw attention to a highly conserved protein polymerization mechanism and shed light on a long-standing biochemical puzzle. We suggest that barnacle cement polymerization is a specialized form of wound healing. The polymerization mechanism common between barnacle cement and blood may be a theme for many marine animal glues.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/212/21/3499/DC1

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INTRODUCTION

The fitness of sessile organisms is dependent on a reliable attachment mechanism. For marine organisms that permanently attach to hard substrates, attachment is typically derived from a secreted adhesive with specific chemical properties. These adhesives are able to displace water, spread and form adhesive bonds with the substrate, as well as coagulate/cross-link, which imparts stability to the adhesive (Waite, 1987). Adhesion in barnacles has elicited considerable scientific attention because of the strength and durability of their adhesive and practical concerns related to marine fouling. Basic biochemical investigations into the nature of barnacle cement have been hindered by its inherent insolubility. Polymerized barnacle cement has not been rendered fully soluble under any conditions (Kamino, 2006). Barnacle cement is composed of approximately 90% protein (Walker, 1972; Naldrett, 1993; Kamino et al., 2000) with the remainder as carbohydrate (1%), lipid (1%) and inorganic ash (4%; 30% of the inorganic ash is calcium) (Walker, 1972). Barnacle cement is an aggregate of at least 10 major proteins, a portion of which have been isolated and sequenced (for reviews see Kamino, 2006; Kamino, 2008). Although progress has been made towards understanding the chemical properties of cement proteins, the biochemical mechanisms of cement polymerization remain largely unknown.

Here, we used evolutionary concepts of conservation and relatedness of essential physiological mechanisms to gain insight into the process of barnacle cement polymerization. Barnacle adhesive insolubility, and hence durability, results from the aggregation and cross-linking of cement proteins (Naldrett, 1993; Naldrett and Kaplan, 1997; Kamino et al., 2000) while in an aqueous saline environment. Stable adherence of barnacles to their substrate represents a vital function, as it enables them to remain on a surface long enough and in sufficient proximity to neighbors to mate and reproduce. We considered the question: what other physiological processes are based on aggregation and cross-linking of proteins in an aqueous saline environment, and are vital to an organism's fitness?

An intuitive answer to this question is blood clotting. The ability to stem blood loss is a life or death matter, as it allows vertebrates and invertebrates to maintain blood volume and minimize bacterial and viral attack. Blood clotting in vertebrates and invertebrates arises from wound signals that trigger a cascade of enzyme activation, protein aggregation and cross-linking events, creating a mechanical barrier and serving to attract other molecules that are part of the

wound healing process. In vertebrates, formation of a stable blood clot is brought about by two interrelated cascades of trypsin-like serine proteases (Davie and Rantoff, 1964; MacFarlane, 1964; Davie et al., 1991; Davie, 2003). Proteolytic activity results in enzyme and structural protein activation, enabling bonding interactions of fibrin monomers. Integrity of assembled fibrin monomers is brought about through transglutaminase-mediated cross-linking (Lorand et al., 1962; Shen and Lorand, 1983; Lorand, 2000; Collet et al., 2005). Invertebrate blood coagulation involves similar enzymatic mechanisms: converging cascades of trypsin-like serine proteases in horseshoe crabs (Muta and Iwanaga, 1996; Iwanaga et al., 1998; Iwanaga, 2002); a transglutaminase (Fuller and Doolittle, 1971; Kopacek et al., 1993; Chen et al., 2005) showing homology to vertebrate transglutaminase (Wang et al., 2001); and proteolytic activation (Durliat and Vranckx, 1981; Madaras et al., 1981; Theopold et al., 2004) in crustaceans.

We tested the broad hypothesis that barnacle cement polymerization is biochemically similar to blood clotting. To do so, we developed a technique that allowed us to collect cement prior to polymerization, in order to detect and experiment with enzymatic activity and display component structural proteins. We describe this technique and provide evidence supporting its validity. We assessed our hypothesis by first considering whether key blood clotting enzymes, trypsin-like serine proteases, are involved in barnacle cement polymerization. Next, we used tandem mass spectrometry to identify clotting proteins that may be present in unpolymerized barnacle cement. The blood clotting enzyme identified, transglutaminase, was verified with western blotting and enzyme assays, the potential source of this enzyme was examined, and the cross-links produced through its activity were identified in polymerized cement. Lastly, we tested whether the enzymatic activity shown in barnacle cement is dependent on Ca^{2+}, a cofactor often essential to trypsin-like serine protease and transglutaminase activity (Folk, 1980; Davie, 2003). The results obtained were used to generate a biochemical model for barnacle cement polymerization.

MATERIALS AND METHODS

Barnacle larval culture, settlement and maintenance

The barnacle *Amphibalanus amphitrite* (previously *Balanus amphitrite*) (Pitombo, 2004) was used. Barnacle larval culture and settlement were conducted at the Duke University Marine Laboratory in Beaufort, NC, following Rittschof et al. (Rittschof et al., 1984a). Barnacle larvae were settled on 7.6 cm× 15.2 cm×0.64 cm glass panels coated with silicone (Dow Corning Silastic T2®, Midland, MI, USA or International Veridian®, Felling, UK) and maintained in the laboratory as described previously (Holm et al., 2005).

X-ray microtomography

Live barnacles were imaged using x-ray microtomography (conducted at the Naval Research Laboratory in Washington, DC). The barnacle shown in Fig. 1A had been settled and grown at the center of a 5 cm polystyrene Petri dish (Falcon no. 351006). The dish was used to hold the barnacle during the scans. The rim of the dish along the path of the x-rays was removed for better resolution. The barnacle was approximately 10.5 mm×9.3 mm along its major and minor axes (parallel and perpendicular to the operculum). The barnacle was not submersed in water during scanning. All x-ray tomograms were taken using a Skyscan model 1172 tomography system with a 1.3 megapixel camera (Skyscan, Kontich, Belgium). Scans were performed using an 80 kV x-ray source voltage setting with 0.45 deg. rotation steps (400 images) and took about 78 min. Image voxel size was 10.4 µm.

Image cone-beam reconstruction was performed using the Skyscan proprietary software (NRECON; www.skyscan.be/products/downloads.htm). Ring artifact correction was applied, but only minimal smoothing and beam hardening corrections were applied. Reconstructed images were further manipulated for viewing using two additional software packages. ImageJ (http://rsb.info.nih.gov/ij/) was used to stack and crop regions of interest, and to remove some reconstruction and rendering artifacts. OsiriX (www.osirix-viewer.com) was used for three-dimensional isosurface renderings. Rendered surfaces were made approximately 25% transparent to reveal the internal structures.

Unpolymerized barnacle cement

For studies utilizing unpolymerized barnacle cement, cement droplets were obtained by hand using a method inspired by Cheung and colleagues (Cheung et al., 1977). Cement production is continuous throughout a barnacle's life (Saroyan et al., 1970), which makes the collection of unpolymerized cement possible. Barnacles were gently removed intact from silicone substrates using a dissecting needle (Hamilton Bell, Montvale, NJ, USA). Barnacles whose base plate broke upon removal were discarded. Following removal, all shell plates (including the base plate) were gently cleaned in deionized water with a cotton swab. Barnacles were then dried with a Kimwipe® and sat in air on a paper towel for 3 h. Allowing time for the barnacles to dry is essential for the formation of defined cement droplets. To stimulate the release of cement, the periphery of the base plate, where cement is normally released during growth (Fig. 1A: the junction of the base plate and parietal plates), was gently pricked in an outward direction with a dissecting needle. Opening the cement channels by removing previously polymerized cement allows for 1–2 µl droplets to form, which can be taken up using a pipettor fitted with a microtip. Very gently squeezing the barnacle between the thumb and finger (compressing the base plate towards the operculum) increased cement volume. After cement collection, unattached barnacles were kept in 10.5 cm glass finger bowls containing 250 ml seawater, and fed daily with 10 ml dense *Artemia* sp. Barnacles were held for up to 2 months. To prevent strong adhesion to the glass bowl during this time, each barnacle was pushed gently (with a finger) to a different location in the bowl daily. Barnacles were used on average once per week for cement collection.

Total protein concentration

A Coomassie protein assay (Bradford, 1976) was used to determine total protein concentration in unpolymerized cement. Total protein assays were conducted using Coomassie Protein Reagent (Pierce Chemical, no. 23200; Rockford, IL, USA). A 0.5 µl sample of unpolymerized cement was taken from each barnacle and immediately added to 15 µl deionized water, vortexed and placed on ice. When all samples were collected, each sample was distributed in three 5 µl aliquots in a 96-well plate containing 250 µl Coomassie reagent. A BSA standard curve (0, 31.25, 62.5, 125, 250, 500 and 1000 µg ml^{-1}) was run with the cement samples. Samples were read at 595 nm on a Molecular Devices SpectraMAX® 190 spectrophotometer (Sunnyvale, CA, USA). Total protein was quantified for 54 barnacles.

Sodium dodecyl sulfate gel electrophoresis (SDS-PAGE)

Immediately after cement collection, reducing sample buffer containing 10% (w/v) SDS and 5% (v/v) 2-mercaptoethanol

(Laemmli, 1970) was added to unpolymerized cement and samples were heated at 100°C for 4 min. Reducing sample buffer was added in excess (80% total volume rather than 50%) to prevent polymerization of cement proteins. Each lane was loaded with 1–3 µl unpolymerized cement. Although the volume of cement varied between gels depending on the application, all lanes within a gel contained the same initial volume of unpolymerized cement. Samples were run on a 4–20% gradient gel (Pierce Precise Precast Protein Gel, no. 25224, 12 lane, 30 µl or no. 25244, 15 lane, 25 µl) along with molecular mass markers (Novagen Trail Mix 10–225 kDa Protein Markers, no. 70980-3; EMD Chemicals Inc., Gibbstown, NJ, USA) at 40 V for 15 min and then at 100 V for 1 h. Gels that were not used for western blotting were stained overnight with Coomassie Blue [0.25% Coomassie Brilliant Blue R-250 (BioRad Electrophoresis grade, no. 161 0400) Hercules, CA, USA], 7.5% acetic acid, 5.0% methanol), then destained in a solution of 25% methanol, 7.5% acetic acid for 30 min, and finally destained in several changes of 7.5% acetic acid for 24–48 h.

Analysis of cement proteins using tandem mass spectrometry

Tandem mass spectrometry served two purposes in this study: (1) it was used to validate our unpolymerized cement collection technique, by determining whether previously sequenced barnacle cement proteins (that are available in the NCBI database) could be identified in our cement samples; and (2) it was used to identify clotting proteins present in unpolymerized barnacle cement, by comparing peptide sequences with those of an organism in which clotting has been well studied – human. Peptides for analysis were produced through direct trypsin digestion of unseparated cement droplets and trypsin digestion of bands isolated from an SDS-PAGE gel. Peptides sequences were compared with those in two NCBI non-redundant databases: (1) Balanoid barnacle [for inclusive genera see Pitombo (Pitombo, 2004)]; and (2) human. Mass spectrometry was conducted at the Protein Mass Spectrometry Facility at the University of Puerto Rico, Rio Piedras Campus.

In solution, trypsin digestion of unpolymerized barnacle cement was conducted by adding 1 µl unpolymerized cement directly to 100 µl of 40 mmol l^{-1} ammonium bicarbonate with 10% acetonitrile (ACN; mass spectrometry grade). Trypsin Gold (1.3 µg; Promega no. V5280; Madison, WI, USA; reconstituted to 1 µg µl^{-1} in 50 mmol l^{-1} acetic acid) was added immediately to each sample. Samples were incubated at 37°C for 15 h, after which time ACN was added to 50% total volume and samples were shipped overnight to the University of Puerto Rico for mass spectrometry analysis.

For in-gel digestion, unpolymerized cement was run on SDS-PAGE under reducing conditions (4–20% gradient gel with 1 µl cement per lane) as described above. Gels were stained overnight (0.25% Coomassie Brilliant Blue R-250, 7.5% acetic acid, 5.0% methanol). Gels were shipped *via* overnight mail to the University of Puerto Rico during staining by placing each gel in a sealed plastic bag with ~50 ml Coomassie Blue stain. Once at the University of Puerto Rico, gels were destained in 40% methanol.

Following initial destaining, individual protein bands detected by Coomassie Blue staining were carefully excised from the gel using a scalpel, destained using 100 mmol l^{-1} ammonium bicarbonate:50% ACN, and then dehydrated in 100% ACN. After removal of ACN by speed-vacuum, the gel slice was re-hydrated in 40 mmol l^{-1} ammonium bicarbonate and 10% ACN. Trypsin (1 mg) was added and incubation was carried out overnight (18 h) at 37°C. The tryptic peptides were eluted from the gel slice by incubating the slice in a solution containing 50% ACN and 5% formic acid for 1 h at room temperature.

Tryptic peptides were loaded onto a Surveyor® HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) and peptides were eluted, using a gradient of ACN (0%–80%) in 0.2% formic acid/H_2O, directly into the electro-spray ionization source. The eluted tryptic peptides were infused into a LTQ mass spectrometer (Thermo Fisher Scientific) for tandem mass spectrometry analysis of the proteins of interest.

Tandem mass spectra were extracted by BioWorks® V. 3.2 (Thermo Fisher Scientific). Charge state deconvolution and deisotoping were not performed. All tandem mass spectrometry samples were analyzed using Sequest® V. 2.7 (ThermoFinnigan, San Jose, CA, USA). Sequest was set to search the Balanoid barnacle and human databases (NCBI non-redundant sub-databases) assuming the digestion enzyme was trypsin. Sequest was searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 2.0 Da. Only those identified peptides that passed selection filters imposed on the database search were taken into consideration for protein identification (cross-correlation higher than 1.5 (+1), 2.0 (+2) or 2.5 (+3); delta score >0.1; 10 or more b and y ions; MS2 intensity of $>5\times10^{-4}$, peptide probability $>E\times10^{-2}$). Additionally, Scaffold® (V. Scaffold-01_07_00, Proteome Software Inc., Portland, OR, USA) was used to validate tandem mass spectrometry based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on tandem mass spectrometry analysis alone were grouped to satisfy the principles of parsimony.

Atomic force microscopy (AFM)

AFM imaging of barnacle cement was used to validate our cement collection technique. AFM was conducted at the Naval Research Laboratory in Washington, DC, using a Veeco Nanoman AFM (Digital Instruments, Dimension 3100; Veeco, Santa Barbara, CA, USA) in tapping mode. All imaging was conducted in air. Imaging in air is appropriate for barnacle cement. While cement is hydrated, hydration levels in ambient environments are not significantly different from those in native barnacle–glue interfaces (Barlow et al., 2009). Images were extracted using WSxM V. 3.0 Beta 11.1 (Horcas et al., 2007).

Primary cement (original cement secreted by the barnacle) was imaged *in situ*, directly on the base of barnacles. Barnacles were gently removed intact from a silicone substrate using a dissecting needle and rinsed briefly in deionized water. The shell plates were dried using a Kimwipe®. For imaging, each barnacle was affixed to a microscope slide in an inverted, level position using sculpting putty.

For AFM of unpolymerized cement after curing, 1 µl unpolymerized cement was deposited onto a 75 mm×25 mm glass microscope slide and immediately covered with a 10 mm×30 mm×1 mm (l×w×d) glass slide (cut to size with a diamond scribe, placed with approximately 5 mm hanging over the edge of the 75 mm×25 mm slide for ease of removal) and placed in seawater to simulate the barnacle–cement–substrate interface. After 48 h, the slide was taken out of seawater, the cover slide removed, and the cured droplet washed lightly in deionized water. The cured droplet was imaged in several regions.

Western blotting for bovine pancreatic trypsin

Following SDS-PAGE (as described previously) proteins were transferred to a PVDF membrane (Millipore Immobilon Psq, 0.2 μm pore size, no. ISEQ 081 00; Billerica, MA, USA). Proteins were transferred at 4°C at 15 V overnight using Tris-glycine transfer buffer (pH 8.3) containing 15% methanol. Gels were stained with Coomassie Blue after blotting to assess protein transfer.

Barnacle cement proteins that had been transferred onto PVDF membranes were blocked with bovine serum albumin (BSA) to reduce non-specific binding and immunostained with antibodies for bovine pancreatic trypsin. TBS buffer (pH 7.6) was used for all immunostaining. Rabbit polyclonal antibody to full-length bovine pancreatic trypsin (1:10,000 dilution) was used as the primary antibody (Novus Biologicals no. NB 600-1277; Littleton, CO, USA). Anti-rabbit polyclonal antibody (1:20,000 dilution), conjugated to horseradish peroxidase, was used as the secondary antibody (Novus Biologicals no. NB7160). Antibodies were detected using a TMB substrate (Vector Laboratories no. SK4400; Burlingame, CA, USA). Endogenous peroxidase activity was not observed on western-blotted proteins. Control staining for non-specific binding was conducted with secondary antibody only. Bovine pancreatic trypsin (TPCK treated, Sigma-Aldrich, no. T1426; St Louis, MO, USA) was western blotted along with barnacle cement proteins as a positive control.

Quantification of trypsin activity

BAPNA (*N*α-benzoyl-DL-arginine 4-nitroanilide; Acros Organics no. 227740010; Geel, Belgium) was used as a trypsin substrate and prepared at 0.044% (w/v) by first dissolving BAPNA in DMSO (dimethyl sulfoxide: 1% v/v) and then adding 50 mmol l^{-1} Tris buffer, pH 8.0. Reaction conditions (pH, incubation temperature, buffer concentration) followed Dougherty (Dougherty, 1996) who optimized reaction conditions for general protease activity in unpolymerized cement from the barnacle *Chthamalus fragilis*.

To assay trypsin activity, 6 μl unpolymerized cement was added to 800 μl BAPNA solution. Samples were vortexed and incubated at 37°C for 1 h. Two controls were run along with cement samples: (1) substrate only – 6 μl Tris buffer (50 mmol l^{-1}, pH 8.0) with 800 μl BAPNA solution, and (2) trypsin positive control – 6 μl trypsin solution (4.63E^{-3} BAPNA units ml^{-1} porcine pancreatic trypsin Type II-S, Sigma-Aldrich no. T7409, in Tris buffer) with 800 μl BAPNA solution. Control samples were vortexed and incubated at 37°C for 1 h along with cement samples. Following incubation, all samples were centrifuged at 6400 ***g*** for 10 min in a Fisher Scientific MicroD centrifuge. Samples were transferred to a quartz semi-micro cuvette (Starna Cells no. 9-Q-10; Atascadero, CA, USA) and optical density at 405 nm (OD_{405}), referenced to Tris buffer alone, was read on a Hewlett Packard 8451A diode array spectrophotometer (Palo Alto, CA, USA). Samples were staggered in approximately eight sample groups (each with controls) so that all samples could be read within 10 min of centrifugation. Statistical analyses were conducted using SigmaStat® V. 3.10.0 (Systat Software Inc., San Jose, CA, USA).

Verification of trypsin activity – barnacle settlement assays

Barnacle cyprids (larval settlement stage) settle in response to peptides with carboxy-terminal arginine or lysine residues, which are generated by trypsin-like serine proteases (Tegtmeyer and Rittschof, 1988; Pettis, 1991). Therefore, settlement in response to small peptides in unpolymerized cement can be used as evidence for the activity of a trypsin-like serine protease in barnacle cement. All statistical analyses for settlement assays were conducted using SigmaStat® V. 3.10.0.

The response of barnacle cyprids to polymerizing cement taken from conspecific adults was tested using a series of settlement assays. Samples of 1, 3 or 6 μl unpolymerized cement were placed in a 5 cm polystyrene Petri dish (Falcon no. 351006) in 1 μl droplets. Immediately afterwards, 5 ml filtered, aged seawater was added to the dish. Approximately 20, newly metamorphosed (day 0) cyprids were added to each dish. Cyprids were allowed to settle for 24 h, over which time they were kept at 27–28°C on a 12 h:12 h light:dark cycle. Settlement treatments (1, 3 and 6 μl) along with controls (clean dish, no cement) were conducted in duplicate. The entire assay was conducted three times.

Settlement in all dishes was quantified after 24 h. Cyprids that had clearly metamorphosed into a juvenile and cyprids that had cemented to the dish but had not yet metamorphosed were counted as settled. Barnacles were killed by adding a drop of formalin to each dish, and settled and unsettled individuals were counted on a dissecting microscope.

Electrophoresis of cement with soybean trypsin inhibitor

Barnacle cement polymerizes rapidly. As polymerization proceeds, the intensity of bands resolvable with gel electrophoresis tends to decrease, since aggregated/cross-linked proteins become too large to enter the gel. If a reagent inhibits an enzyme involved in cement polymerization, then after a given incubation time the protein banding pattern should be stronger with than without inhibitor. The effect of trypsin inhibitor (soybean, Sigma-Aldrich no. 93620) on cement polymerization was tested using SDS-PAGE (under reducing conditions). A 1 μl sample of unpolymerized cement was added to 2 μl of trypsin inhibitor solution (250 ng ml^{-1} initial concentration). As a control, 1 μl unpolymerized cement was added to 2 μl deionized water. Samples were vortexed and incubated on ice for 2 min. After 2 min incubation, reducing sample buffer was added, samples were heated, run on SDS-PAGE, and stained/destained as described above. Four replicate trypsin inhibitor treatments and four deionized water controls were conducted. Gels were photographed after destaining with a digital camera. To allow comparison of protein band intensity among lanes, each gel lane was analyzed for pixel intensity using Scion Image® V. Alpha 4.0.3.2 (Scion Corporation, Frederick, MD, USA). The intensity of prominent protein bands (specifically those at 175, 125, 100, 90, 80, 68, 52, 38, 32, 28 and 25 kDa) was compared statistically between trypsin inhibitor treatments and deionized water controls using SigmaStat® V. 3.10.0.

Western blotting for human transglutaminase (factor XIII A1 subunit)

Tandem mass spectrometry identified the presence of transglutaminase (factor XIII A1 subunit precursor) in barnacle cement. Western blotting was used to verify the mass spectrometry results. Cement samples (1 μl) were denatured in 20 μl reducing sample buffer containing 10% (w/v) SDS and 5% (v/v) 2-mercaptoethanol and heated at 100°C for 4 min. After denaturing treatment, samples were sent *via* overnight mail to the University of Puerto Rico for western blotting.

At the University of Puerto Rico, samples were run on a 10% acrylamide gel at four sample concentrations: the full cement sample (20 μl containing 1 μl cement), 3 μl sample, 2 μl sample and 1 μl sample. The gel was run at 60 V for 30 min and then 150 V until the dye front had run out of the gel. Cement proteins were transferred onto a nitrocellulose membrane in Tris-glycine transfer buffer containing 16% methanol. Protein transfer was conducted at 300 mA for 2 h at 4°C. Following protein transfer, the nitrocellulose

membrane was placed in blocking solution (containing 5% non-fat dry milk) for 1 h. Rabbit polyclonal antibody to human factor XIII A subunit (GeneTex Inc., no. GTX72947; Irvine, CA, USA) was used as the primary antibody at 1:500 dilution. Goat anti-rabbit HRP-conjugated antibody (Chemicon, Millipore) was used as the secondary antibody at 1:2000 dilution. Following staining, the membrane was incubated in ECL Plus (GE Healthcare, Little Chalfont, Bucks, UK) for 2 min and then exposed to film. Control staining for non-specific binding was conducted with secondary antibody only. Staining was optimal when the full cement sample was used.

Quantification of transglutaminase activity

Transglutaminase activity in unpolymerized cement was quantified using a transglutaminase assay kit (Sigma-Aldrich no. CS1070). Assays were based on the reaction of transglutaminase with a cadaverine-coated 96-well plate; 1 µl unpolymerized cement was used for each test well. Cement was first added to assay buffer in a microfuge tube and vortexed. Cement in assay buffer was held on ice until all cement samples had been collected, at which time assay buffer containing cement was transferred to plate wells. Substrate only (no enzyme present) and positive controls (2 mU ml^{-1} transglutaminase from guinea pig liver), each in triplicate, were run along with barnacle cement samples. Since quantification of transglutaminase was based on peroxidase-conjugated streptavidin, and we have observed endogenous peroxidase activity in barnacle cement (Dickinson, 2008) (and G.H.D., B.O. and D.R., in preparation), optical density at 450 nm (OD_{450}) values were corrected for barnacle endogenous peroxidase activity. To enable this correction, substrate only and cement were run in wells without streptavidin–peroxidase added. Absorbance due to barnacle cement endogenous peroxidase was calculated as the OD_{450} value for cement without streptavidin–peroxidase minus mean OD_{450} for substrate only without streptavidin–peroxidase. The peroxidase correction for each individual was subtracted from the OD_{450} for cement run with streptavidin–peroxidase.

Hemocytes in cement droplets

The presence of hemocytes (a potential source of transglutaminase) in cement droplets was verified and quantified using a hemocytometer (American Optical Corporation, no. 1483; Greenwich, CT, USA). Heparin (ammonium salt, porcine intestinal mucosa, Sigma no. H 0880) was used as an anticoagulant to reduce the rate of cement polymerization. A 1 µl droplet of 1 mg ml^{-1} heparin was first placed onto the counting grid; 1 µl cement was added to the heparin solution immediately after removal from the barnacle base and a coverslip was placed over the cement–heparin solution. The hemocytometer was placed on a compound microscope under phase contrast optics and photographed using a digital camera. Cell counts were made by reviewing these images on a computer monitor. Cells were classified as hyaline, granular or 'agglomerations' based on morphology as described in Bauchau (Bauchau, 1981) and Hose et al. (Hose et al., 1990). Cells were scored as agglomerations when they appeared as a clumping of several smaller cells. Hemocytes from six different cement droplets were counted.

Identification of ε-(γ-glutamyl)lysine cross-links in barnacle cement

Transglutaminase activity results in the formation of ε-(γ-glutamyl)lysine cross-links (Pisano et al., 1968). The chemical method described by Pisano and colleagues (Pisano et al., 1969) was used to identify these cross-links in barnacle cement. This method is able to distinguish Lys that is bound specifically in ε-(γ-glutamyl)lysine cross-links from Lys that is not cross-linked. Proteins are subject to cyanoethylation and then acid hydrolysis. Lys residues with a free amino group (non-cross-linked) are converted to *N*-carboxyl derivatives upon cyanoethylation and are not detected during amino acid analysis. Lys residues that are cross-linked are released as free Lys.

Polymerized cement was obtained by scrapping off and pooling the soft, opaque cement that is formed by some barnacles when attached to silicone substrates (Wiegemann and Watermann, 2003; Holm et al., 2005). After collection, cement was rinsed with deionized water and lyophilized. A total of 11.3 mg of lyophilized cement was collected. Roughly half of the collected cement (5.5 mg) was subject to cyanoethylation, whereas the remaining 5.8 mg was left untreated. For cyanoethylation, 20 µl triethylamine and 200 µl acrylonitrile were added to the 5.5 mg cement sample, which was then incubated at 37°C for 104 h in a sealed ampoule under N_2, as described by Pisano and colleagues (Pisano et al., 1969). These reaction conditions have been optimized for clotted fibrin by Pisano and coworkers (Pisano et al., 1969), ensuring that the cyanoethylation reaction would go to completion and that all free Lys would react. The cyanoethylated sample and the untreated cement sample were sent to the University of California at Davis Protein Chemistry Facility, where acid hydrolysis (6 mol l^{-1} HCl/0.1% phenol, 110°C, 24 h) and amino acid quantification were conducted.

Electrophoresis of cement with Ca^{2+} chelators

The effect of divalent cation chelators on cement polymerization was tested using SDS-PAGE (under reducing conditions), as described above for soybean trypsin inhibitor. EGTA (Sigma-Aldrich no. E4378) and EDTA (Fisher Chemical, no. BP118) were used. The initial concentration of EGTA and EDTA solutions was 1 mg ml^{-1} (stirred for at least 30 min before use). Three replicate EDTA and EGTA treatments were conducted. As described for trypsin inhibitor, the intensity of prominent protein bands (specifically those at 175, 125, 100, 90, 80, 68, 52, 38, 32, 28 and 25 kDa) was compared statistically between EDTA or EGTA treatments and deionized water controls using SigmaStat® V. 3.10.0.

RESULTS

Collection of unpolymerized cement

We developed a novel method for the collection of barnacle cement in the unpolymerized state. Barnacles were removed intact from 'easy release' silicone panels and dried in air for several hours. Polymerized cement at the junction of the base and lateral plates (Fig. 1A), where cement is released upon growth, was gently removed with a dissecting needle, stimulating the release of unpolymerized cement (Fig. 1B, arrow). Cement droplets averaged 1 µl in volume, but were observed up to 6 µl. When initial cement volume was low (<1 µl), droplet volume was increased by squeezing the barnacle gently, compressing the base plate towards the operculum. If shell plates were not given sufficient time to dry, droplets adhered to the moist shell and were difficult to take up. Cement was successfully collected from most barnacles on most days. Barnacles were used repeatedly for cement collection (on average once per week for up to 2 months); they very rarely died following the collection procedure.

Multiple independent techniques were used to verify that the secretion collected was in fact barnacle cement. Consistent with a proteinaceous cement that polymerizes, unpolymerized cement collected as described here contained a large amount of protein

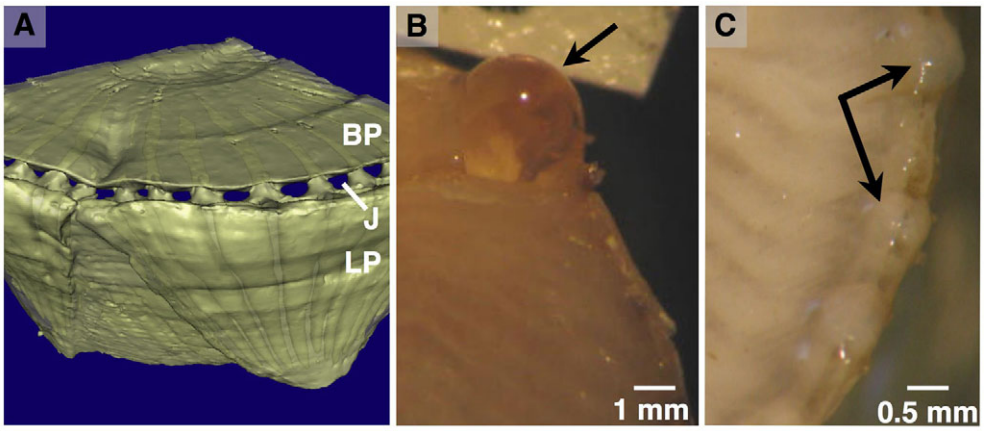

Fig. 1. Barnacle cement release and polymerization. (A) X-ray microtomograph of a live barnacle (*Amphibalanus amphitrite*) showing the junction (J) of the base plate (BP) and lateral plates (LP), where cement is released through ducts during growth. (B) Manual removal of polymerized cement from cement ducts resulted in the release of unpolymerized cement, which could be collected in microliter quantities. The barnacle had been gently removed from a silicone substrate. (C) Polymerized cement following release.

(10.3±0.8 μg μl^{-1}; mean±s.e.m.) and polymerized (Fig. 1C). Unpolymerized cement contained all of the previously sequenced barnacle cement proteins and structural proteins (SIPC), as identified by tandem mass spectrometry (Table 1). Furthermore, when unpolymerized cement was cured in seawater between plates, its ultrastructure was in accord with that of cement imaged directly on the base of a barnacle (i.e. primary cement) (Fig. 2). AFM revealed insoluble fibrous motifs, indicative of progressive cross-linking of the cement. Fibers were typically 2–25 nm in diameter (based on AFM height measurements) and occurred in a tightly interlocking network, consistent with previous electron microscope and AFM studies of cement (Wiegemann and Watermann, 2003; Sullan et al., 2009).

Trypsin-like serine protease activity

We assessed the presence and activity of trypsin-like serine proteases in barnacle cement by analysis of gel electrophoresis banding patterns, western blotting, enzymatic assays using a trypsin-specific substrate, barnacle larval settlement assays, and tests with trypsin inhibitor. Gel electrophoresis (SDS-PAGE) was used to separate cement proteins based on molecular mass. SDS-PAGE of unpolymerized barnacle cement revealed multiple doublet bands and an abundance of small peptides, visible as a cloud at the bottom of the gel (Fig. 3A). Silver staining of SDS-PAGE gels resulted in multiple clear protein bands of less than 10 kDa (G.H.D., B.O. and D.R., in preparation). Although doublet bands can arise from various chemical modifications, their presence is often associated with protease clipping and activation of native proteins (Ojha, 1996; Biro et al., 2003). In such cases, the inactive form of the protease appears as the larger band of the doublet, the activated form appears as the smaller band of the doublet, and small peptides resulting from proteolytic cleavage migrate to the bottom of the gel.

A vertebrate trypsin-like protein, occurring as a single band at 90 kDa or a doublet at 90 and 80 kDa, was identified in unpolymerized barnacle cement by western blotting (Fig. 3A). Immunoreactivity was shown towards polyclonal bovine pancreatic trypsin antibody. Western blotting results were clear and readily reproducible. Cement proteins that had been incubated with secondary antibody only did not stain.

We verified trypsin-like serine protease activity in unpolymerized barnacle cement through assays with BAPNA, a trypsin-specific, arginine ester substrate (Fig. 3B). As shown in Fig. 3B, mean OD_{405} for the unpolymerized cement group (N=20 individuals) differed significantly from the substrate only control group (no enzyme present) (rank sum test: P=0.006). Barnacle larval settlement assays were also used to provide evidence of trypsin-like serine protease activity, because these assays detect serine protease-generated peptides (Tegtmeyer and Rittschof, 1988; Pettis, 1991). The presence of cement droplets in assay dishes caused a dramatic increase in cyprid settlement at all cement volumes tested, suggesting the release of serine protease-generated peptides (Fig. 3C). Settlement did not occur directly on the cement droplets. Total percentage settlement differed significantly between 1, 3 and 6 μl cement and control groups (Kruskal–Wallis one-way ANOVA on ranks: P=0.009), and all treatment groups (1, 3 and 6 μl) showed a greater total percentage settlement than did the control group (SNK pairwise comparison: P<0.05).

The involvement of trypsin-like protease(s) in barnacle cement polymerization was shown using polymerization assays with soybean trypsin inhibitor. Cement was incubated in the presence of soybean trypsin inhibitor or deionized water (control) for 2 min and then run on SDS-PAGE. Following staining, the intensity of prominent protein bands (those labeled in Fig. 3A) was determined using image analysis software. It was expected that in controls, the

Table 1. Relevant Balanoid barnacle proteins identified by tandem mass spectrometry

Protein name	Accession no.	Molecular mass (kDa)	No. of peptides
Cement protein-100k (*Megabalanus rosa*)	9967109	113.628	47
Cement protein-19k (*Balanus albicostatus*)	97974213	17.331	13
Cement protein-19k (*Balanus improvisus*)	97974218	16.836	14
Cement protein-20k (*Megabalanus rosa*)	11559270	22.447	4
Cement protein-19k (*Megabalanus rosa*)	97974207	19.500	6
CB-1=cement protein cyanogen bromide peptide 1 (*Megabalanus rosa*)	1835875	3.300	3
Settlement inducing protein complex (*Balanus amphitrite*)	71361896	170.702	97

Analyzed peptides were from trypsin-digested whole unpolymerized cement. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides.

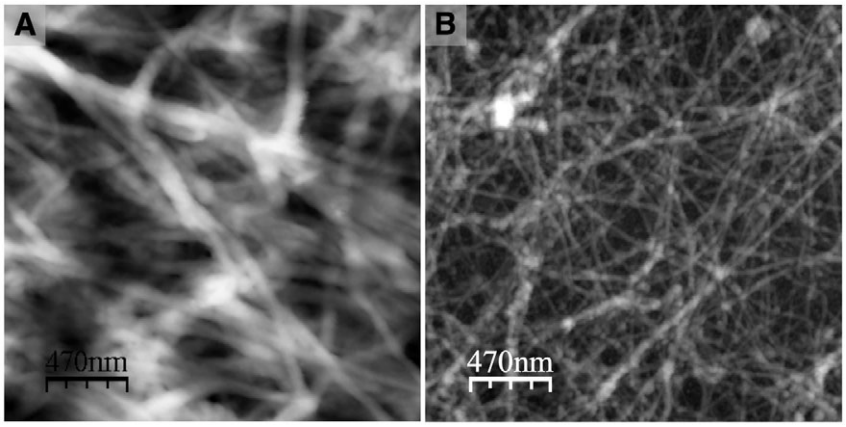

Fig. 2. Atomic force microscopy (AFM) images of polymerized barnacle cement. (A) Cement was imaged *in situ*, directly on a barnacle base. (B) Unpolymerized cement, collected as shown in Fig. 1, was cured under seawater while sandwiched between glass slides. In both cases, AFM imaging revealed a fibrous ultrastructure.

intensity of protein bands involved in polymerization would decrease as cement polymerization proceeds, since aggregated/cross-linked proteins become too large to enter the gel. Mean and s.e.m. intensity values for four replicate trypsin inhibitor treatments and deionized water controls are shown in Fig. 4. The intensity of the 90, 68, 52, 38 and 32 kDa protein bands differed significantly between trypsin inhibitor treatments and controls (two-tailed *t*-tests: $P<0.05$), with trypsin inhibitor showing greater intensity in each case. This result indicates that trypsin inhibitor impeded the aggregation/polymerization of these five proteins. Other protein bands (specifically the intense bands at 175 and 28 kDa) did not differ between trypsin inhibitor treatments and deionized water controls. Using this assay we were not able to discern a difference in the intensity of small peptides between trypsin inhibitor treatments and controls, nor were we able to track changes in the intensity of doublet members as polymerization proceeded.

Transglutaminase activity

We used tandem mass spectrometry to identify clotting proteins present in unpolymerized barnacle cement by comparing peptide sequences with the human protein database. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides. The catalytic subunit of human 'fibrin stabilizing factor', a transglutaminase (factor XIII A1 subunit precursor; accession number NP_000120.1), was identified (Fig. 5A,B; for peptide fragmentation and details on the tandem mass spectrometry method see supplementary material Fig. S1). The presence of this protein was validated by western blotting using anti-human factor XIII A subunit antibody (Fig. 5C). As shown in Fig. 5C, the transglutaminase occurred at 75 kDa. Cement proteins that had been incubated with secondary antibody only did not stain.

Transglutaminase activity assays were used to determine whether the identified transglutaminase showed enzymatic activity (Fig. 5D). Transglutaminase activity was found for all individuals assayed. As shown in Fig. 5D, mean OD_{450} for the unpolymerized cement group (N=19 individuals) differed significantly from the substrate only control group (no enzyme present) (two-tailed *t*-test: P=0.006).

Hyaline cells are the principal source of transglutaminase in crustaceans (Omori et al., 1989; Martin et al., 1991). We assessed

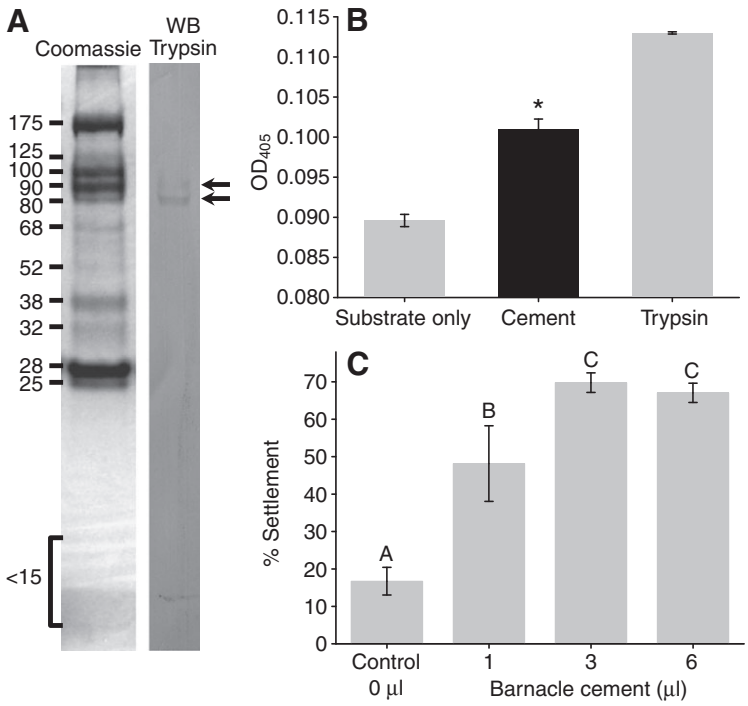

Fig. 3. Trypsin-like serine protease in unpolymerized barnacle cement. (A) Left: SDS-PAGE (reducing conditions) of unpolymerized barnacle cement showing multiple doublet bands (Coomassie stained). Prominent bands are labeled with molecular mass in kDa. Right: a mammalian trypsin-like protein in unpolymerized barnacle cement as shown by western blot (WB) using polyclonal bovine pancreatic trypsin antibody. Staining bands occur at 90 and 80 kDa and are indicated by arrows. (B) Verification of trypsin activity in unpolymerized barnacle cement, using BAPNA. Mean optical density at 405 nm (OD_{405}) (and s.e.m.) for unpolymerized cement (N=20 individuals), substrate only (no enzyme present) and trypsin control ($4.6E^{-3}$ BAPNA units per ml porcine trypsin) is shown. *Significant difference between the substrate only and cement groups (rank sum test: P=0.006). (C) Barnacle larval settlement in the presence of polymerizing barnacle cement. A positive settlement response is evidence of trypsin-like serine protease activity, because peptides generated by trypsin-like protease activity induce barnacle settlement. Settlement assays were conducted for 24 h with newly metamorphosed cyprids. Means and s.e.m. are shown. Groups marked with different letters are significantly different as shown by SNK pairwise comparison. N=5 assays per cement volume.

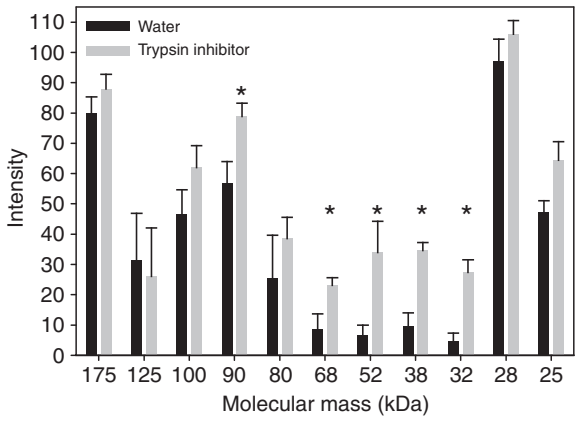

Fig. 4. Inhibition of barnacle cement polymerization with soybean trypsin inhibitor. Unpolymerized cement was incubated with soybean trypsin inhibitor or deionized water for 2 min and then run on SDS-PAGE. Mean intensity (+s.e.m.) for the protein bands labeled in Fig. 3A is shown (N=4 replicate lanes). *Significant difference in band intensity between the trypsin inhibitor and deionized water groups (t-tests: P<0.05).

the presence of hyaline cells in barnacle cement by examining unpolymerized cement droplets under phase contrast optics (Fig. 5E). Six, 1 µl cement samples were scored for cell density and composition. Hemocytes were observed in all cement samples, at a mean (±s.e.m.) density of $(1.73\pm0.21)\times10^2$ cells µl^{-1}. The vast majority of hemocytes were hyaline cells (84.7±2.2% per sample; mean±s.e.m.). Granular cells (likely semi-granular) and agglomerations were much less common at 10.1±1.5% and 8.3±2.0% per sample, respectively (means±s.e.m.).

To confirm that observed transglutaminase activity is involved in cement polymerization, we identified the presence of ε-(γ-glutamyl)lysine cross-links in polymerized barnacle cement. ε-(γ-Glutamyl)lysine cross-links (Fig. 6) result specifically from transglutaminase activity. Polymerized cement was scraped from several barnacles and subjected to cyanoethylation and acid hydrolysis or acid hydrolysis only. As described by Pisano and colleagues (Pisano et al., 1969), cyanoethylation differentiates cross-linked from uncross-linked Lys and explicitly identifies ε-(γ-glutamyl)lysine cross-links. Cross-linked Lys residues that have been subjected to cyanoethylation are liberated as free Lys upon acid hydrolysis, whereas uncross-linked Lys are converted to *N*-carboxyl Lys derivatives. Optimized reaction conditions were employed, ensuring that the cyanoethylation reaction had gone to completion. Free Lys was detected after cyanoethylation treatment, indicating that a portion of the Lys residues in barnacle cement had been bound in ε-(γ-glutamyl)lysine cross-links. Comparing the amount of free Lys in cyanoethylation-treated samples with that in untreated samples provides an estimate of the percentage of Lys residues bound in ε-(γ-glutamyl)lysine cross-links: 5% of Lys residues in barnacle cement were bound in cross-links, based on 0.046 nmol Lys per nmol Arg detected in the treated sample and 0.902 nmol Lys per nmol Arg detected in the untreated sample.

Ca^{2+} dependence

Serine protease and transglutaminase activity is typically Ca^{2+} dependent (Folk, 1980; Davie, 2003). We tested the Ca^{2+} dependence of barnacle cement enzymes through SDS-PAGE polymerization assays with EDTA and EGTA (divalent cation chelators). Assays were carried out and analyzed in the same manner as described for trypsin inhibitor. Means and s.e.m. for three replicate EDTA and EGTA treatments and four replicate deionized water controls are shown in Fig. 7. The intensity of protein bands in EDTA and EGTA treatments varied considerably among replicates. For EDTA, the intensity of the 38 and 25 kDa protein bands differed significantly from the deionized water controls (two-tailed t-tests: P<0.05) with EDTA showing greater intensity in both cases. Other protein bands did not differ significantly from controls. For EGTA treatments, none of the protein bands analyzed showed a significant difference from deionized water controls.

DISCUSSION

We set out to determine the biochemical mechanism of barnacle cement polymerization by testing the hypothesis that cement polymerization is biochemically similar to blood clotting. Based on experimental evidence, we suggest that barnacle cement polymerization involves proteolytic activation of enzymes and structural precursors, transglutaminase cross-linking, and assembly of fibrous proteins. Peptides and/or epitopes homologous to vertebrate trypsin and transglutaminase were identified in barnacle cement with tandem mass spectrometry and/or western blotting. The peptides generated during proteolytic activation functioned as signal molecules, linking a molecular level event (protein aggregation) to a behavioral response (barnacle larval settlement).

Collection of unpolymerized cement

Unpolymerized barnacle cement was collected using a method inspired by Cheung et al. (Cheung et al., 1977). Unlike previously described collection methods, unpolymerized cement could be collected in microliter quantities enabling a wide-range of biochemical and physical assays to be carried out. Most importantly, cement could be collected prior to polymerization, which renders certain cement proteins completely insoluble. Barnacle cement collected in the manner described here was proteinaceous and polymerized. We confirmed that the collected secretion was indeed barnacle cement using tandem mass spectrometry and AFM. We suspect that this cement collection technique will be broadly applicable to studies of barnacle adhesion, biomimetics and the prevention of marine fouling.

Trypsin-like serine protease activity

We tested whether trypsin-like serine protease(s) were involved in barnacle cement polymerization using various techniques. In well-studied organisms (i.e. all vertebrates, horseshoe crabs), the formation of a stable blood clot is brought about by two closely interrelated proteolytic cascades (reviewed by Davie et al., 1991; Muta and Iwanaga, 1996; Iwanaga, 2002; Davie, 2003). Trypsin-like serine proteases are converted to their active form by limited proteolysis and in turn activate the next protease in the cascade. The trypsin-like serine proteases comprising the blood coagulation cascade in vertebrates are all related to pancreatic trypsin (Neurath, 1984; Neurath, 1986) and range in molecular mass from 50 to 160 kDa (Davie and Fujikawa, 1975). Variations in molecular mass are due to amino-terminal extensions, which mediate interactions with substrates, cofactors and inhibitors (Patthy, 1993; Neurath, 1999).

Gel electrophoresis (SDS-PAGE) of unpolymerized cement yielded a pattern consistent with (although not exclusive to) protease clipping and activation of native proteins (Ojha, 1996; Biro et al., 2003). Multiple doublet bands and an abundance of small peptides were observed. Based on the results of trypsin activity assays, we believe that this pattern is due to proteolytic activity. In this case, inactive proteins would occur as the larger member of the doublet,

A

Transglutaminase, factor XIIIA peptides	*z*	XC score	DeltaCn
R.SWSYGQFEDGILDTCLYVMDRAQMDLSGR.G	3	2.66	0.10
R.EEYVLNDIGVIFYGEVNDIK.T	**2**	**2.29**	**0.12**

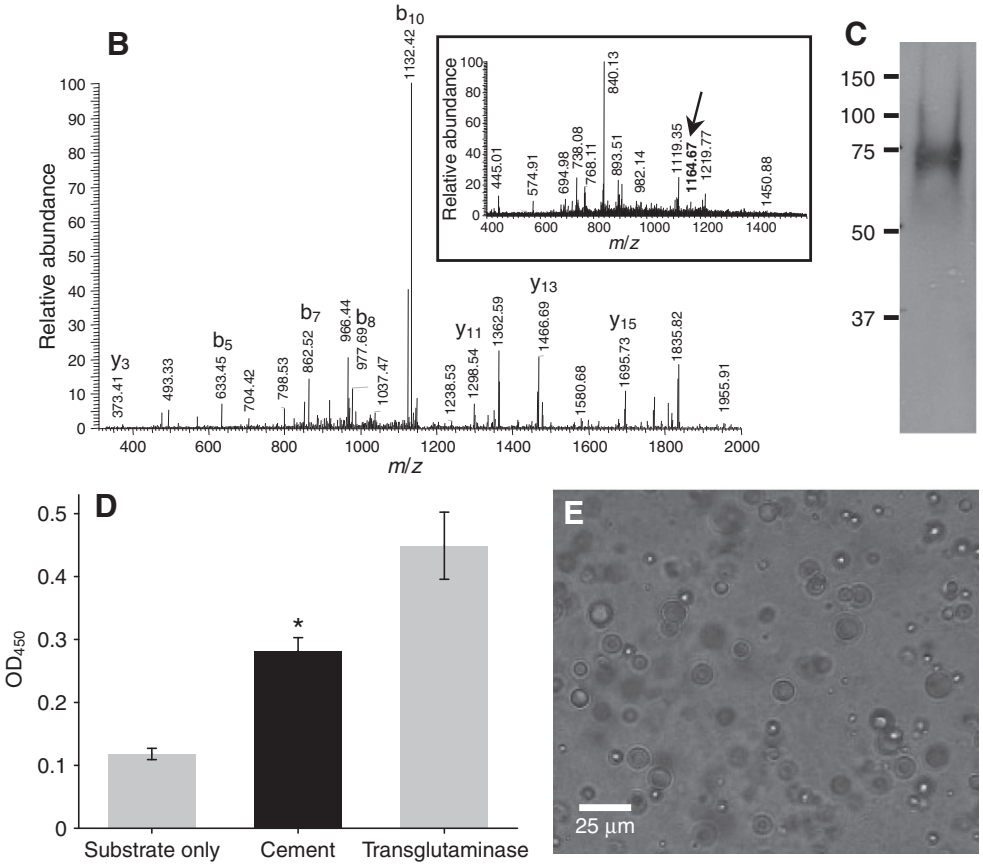

Fig. 5. Transglutaminase in unpolymerized barnacle cement. (A,B) Tandem mass spectrometry (MS/MS) identification of the catalytic subunit of human fibrin stabilizing factor, a transglutaminase (factor XIII A1 subunit precursor, accession number NP_000120.1). Identification was made by comparing the MS/MS spectra with the human database. (A) Identified peptides. XC score, cross-correlation score; deltaCn, difference between XC scores for the best and second best match. (B) Mass spectrometry (MS; inset) and MS/MS spectra for the factor XIII A1 peptide shown in bold. Arrow indicates the precursor mass in the MS spectra. b and y ions obtained upon fragmentation of the precursor ion by collision-induced dissociation are shown. For peptide fragmentation and details of the tandem mass spectrometry method see supplementary material Fig. S1. (C) Validation by western blot analysis using anti-human factor XIII A subunit antibody. Markers in kDa. (D) Verification of transglutaminase enzyme activity in unpolymerized barnacle cement. Mean OD_{450} (and s.e.m.) for unpolymerized cement (N=19 individuals), substrate only (no enzyme present) and transglutaminase control (2 mU ml^{-1} guinea pig transglutaminase) is shown. *Significant difference between the substrate only and cement groups (t-test: P=0.006). (E) Hyaline cells in unpolymerized barnacle cement (phase contrast optics), the principal source of transglutaminase in crustaceans.

activated proteins as the smaller member of the doublet, and proteolytic clips would migrate to the bottom of the gel. One of these doublet bands (at 90 and 80 kDa) showed cross-reactivity toward bovine pancreatic trypsin, further supporting our assertion that the SDS-PAGE banding pattern is due to proteolytic activity. The fact that cross-reactivity was shown toward a vertebrate antigen is indicative of protein sequence (epitope) conservation across very distantly related species.

Polymerization assays with soybean trypsin inhibitor were used to determine whether the observed trypsin-like activity was directly involved in the polymerization of barnacle cement. After only 2 min incubation, 5 out of the 11 protein bands analyzed showed a statistically significant difference in intensity between trypsin inhibitor treatments and deionized water controls. This included all but one protein band within the 90–32 kDa range. In each case protein band intensity was greater when trypsin inhibitor was present than in its absence (deionized water controls). These data suggest that trypsin inhibitor impeded the aggregation/polymerization of specific cement proteins, and therefore that trypsin-like enzyme activity plays a role in cement polymerization. We predict that during cement polymerization trypsin-like serine protease(s) activate structural and proteolytic precursors, maximizing the potential for bonding interactions with other structural proteins and with the surface. Activation of structural proteins would facilitate bonding interactions that have previously been shown to contribute to cement insolubility [i.e. hydrophobic interactions and disulfide bonds (Barnes and Blackstock, 1976; Naldrett, 1993; Naldrett and Kaplan, 1997; Kamino et al., 2000; Kamino, 2008)]. The proteins whose intensity was not affected by trypsin inhibitor: (1) may polymerize at a rate which is not detectable by the current assay (either too fast or too slow), (2) may polymerize by a mechanism independent of trypsin activity, or (3) do not polymerize and are not involved in cement polymerization.

Due to the difficulties associated with acquiring unpolymerized cement, few studies have focused on the enzymes involved in cement polymerization. Dougherty (Dougherty, 1996; Dougherty, 1997) considered protease activity in unpolymerized cement from the barnacle *Chthamalus fragilis*. Consistent with our findings, protease activity was shown in *C. fragilis* cement. Activity was enhanced in the presence of Ca^{2+} ions. Although Dougherty (Dougherty, 1996; Dougherty, 1997) does not provide evidence for a trypsin-like serine protease, he does suggest activity of a zinc metalloprotease with the same substrate preference as a trypsin-like serine protease (carboxy-terminal basic amino acids).

Proteases that cleave peptide bonds at the carboxy-terminus of basic amino acids (trypsin-like serine proteases) result in peptides that are signal molecules in a variety of systems (reviewed by Rittschof, 1990; Rittschof, 1993; Rittschof and Cohen, 2004). These

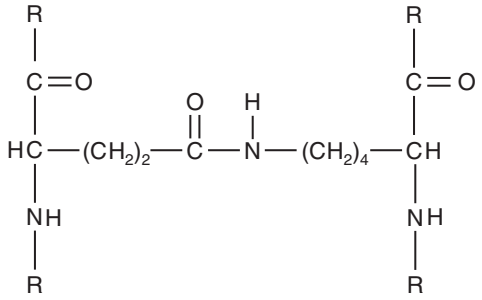

Fig. 6. ε-(γ-Glutamyl)lysine cross-link, which results from transglutaminase activity; 5% of Lys residues in barnacle cement were shown to be bound in ε-(γ-glutamyl)lysine cross-links.

systems include prey location by gastropods, hermit crab shell location, larval release in decapod crustaceans, mammalian complement cascade, and induction of larval settlement in barnacles. Synthetic analogues of mammalian C3a and C5a arginine terminal peptides in the form of basic–basic and neutral–basic dipeptides and neutral–neutral–basic tripeptides were shown to induce barnacle settlement (Tegtmeyer and Rittschof, 1988; Pettis, 1991). Removal of the carboxy-terminal amino acid with carboxypeptidase A destroys biological activity (Rittschof et al., 1984b). The importance of a basic amino acid at the carboxy-terminus, as well as the ability to produce signal molecules by the addition of exogenous trypsin (Rittschof, 1980; Forward et al., 1987; Rittschof et al., 1990), suggests that these peptides are generated by trypsin-like proteases.

In this study, barnacle cyprid settlement in response to small peptides released from polymerizing cement was used as evidence for the activity of a trypsin-like serine protease. Cyprids showed a clear pheromone-induced settlement response when in the presence of polymerizing cement, with the proportion of settled cyprids being dramatically higher in cement treatments than in controls. Evidence that settlement was in response to a waterborne peptide pheromone rather than a substrate-bound cue was provided by the position of cyprid settlement (cyprids did not settle directly on cement droplets, which may contain surface-bound settlement cues), and through settlement assays conducted on cement separated by molecular mass (Dickinson, 2008) (and G.H.D., B.O. and D.R., in preparation). This suggests that the process of cement polymerization, which is necessary for barnacle growth, results in the release of peptide pheromones that induce cyprid settlement and attract predators. Hence, barnacle growth and the associated release of cement may play an important role in structuring intertidal communities.

Transglutaminase activity

Tandem mass spectrometry was used to identify clotting proteins present in unpolymerized barnacle cement by comparing peptide sequences with the human protein database. Remarkably, the catalytic subunit of human 'fibrin stabilizing factor', a transglutaminase, was identified and validated by western blotting. This protein was nearly the same molecular mass in barnacle cement as in human plasma (75 kDa in barnacle cement *versus* 83 kDa in human plasma), despite roughly a billion years of separation between these two species (Brooke, 1999). We verified the activity of transglutaminase in unpolymerized barnacle cement with transglutaminase enzymatic activity assays. Furthermore, we showed the presence of ε-(γ-glutamyl)lysine cross-links, which are produced by transglutaminase activity, in polymerized barnacle cement.

Transglutaminase plays a key role in vertebrate and invertebrate blood coagulation, as it increases clot integrity and resistance to lysis (Shen and Lorand, 1983; Lorand, 2000; Theopold et al., 2004). Evolutionarily, covalent cross-linking of clottable blood protein by transglutaminase pre-dates the proteolytic clotting cascades (Lorand et al., 1966; Laki, 1972). The use of a transglutaminase in covalent cross-linking of clottable protein is widespread, occurring in all vertebrates (Doolittle et al., 1963) and many invertebrates (reviewed by Osaki and Kawabata, 2004; Theopold et al., 2004; Jiravanichpaisal et al., 2006). Transglutaminase-mediated cross-linking is well documented in crustaceans (Fuller and Doolittle, 1971; Madaras et al., 1981; Kopacek et al., 1993; Hall et al., 1999; Chen et al., 2005) and, as in vertebrates, results in an ε-(γ-glutamyl)lysine cross-link between clotting proteins (Fuller and Doolittle, 1971). Unlike vertebrate transglutaminase, activation of crustacean transglutaminase does not appear to be dependent on proteolytic activity (Fuller and Doolittle, 1971). Rather, during clotting crustacean transglutaminase is released from hemocytes, primarily upon lysis of hyaline cells (Martin et al., 1991), and is activated by Ca^{2+} (Sritunyalucksana and Soderhall, 2000). Consistent with cellular release of transglutaminase, we observed hemocytes in unpolymerized cement with the vast majority being hyaline cells.

Transglutaminase cross-linked vertebrate blood clots are insoluble in urea, a property diagnostic of transglutaminase activity (Laki,

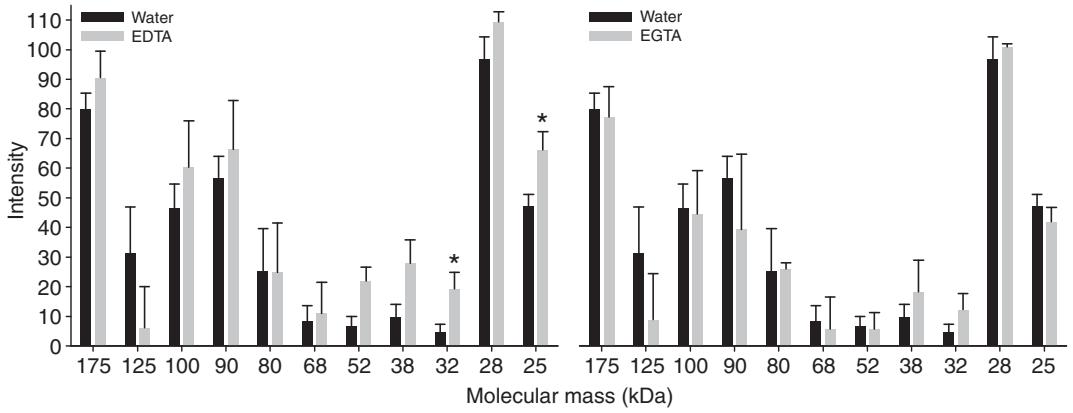

Fig. 7. The effect of EDTA (left) and EGTA (right) on barnacle cement polymerization. Unpolymerized cement was incubated with EDTA, EGTA or deionized water for 2 min and then run on SDS-PAGE. Mean intensity (+s.e.m.) for the protein bands labeled in Fig. 3A is shown (N=4 replicate lanes for deionized water, N=3 replicate lanes for EDTA and EGTA). *Significant difference in band intensity between the EDTA and deionized water groups (t-tests: P<0.05).

1972). Similarly, polymerized barnacle cement has been shown to be insoluble in 8 mol l^{-1} urea (Kamino et al., 1996; Naldrett and Kaplan, 1997). Covalent cross-linking of barnacle cement proteins brought about by a transglutaminase likely plays a key role in the inherent insolubility of barnacle cement, and may explain why polymerized cement is not fully soluble even under strong denaturing treatments. The extent of cross-linking we observed [5% of Lys residues bound in an ε-(γ-glutamyl)lysine cross-links] is consistent with that of human fibrin [2.1% of Lys cross-linked (Hoffner et al., 2009)] and lobster clotting protein [4–6 cross-links per molecule (Fuller and Doolittle, 1971)], and should contribute substantially to cement integrity. Transglutaminase cross-linking of adhesive proteins may not be unique to barnacle cement. Indeed, Epstein and Nicholson (Epstein and Nicholson, 2006) suggest that adhesive polymerization in fungi and oomycetes is likewise brought about by transglutaminase activity.

Ca^{2+} dependence

The serine proteases involved in coagulation systems are typically Ca^{2+} dependent (Davie, 2003). The activity of a protease previously identified in cement of the barnacle *C. fragilis* was shown to be enhanced in the presence of Ca^{2+} ions (Dougherty, 1996; Dougherty, 1997). Transglutaminases in both vertebrates and invertebrates are Ca^{2+} dependent (Lorand et al., 1962; Fuller and Doolittle, 1971; Folk, 1980). The results of our cement polymerization assays with EDTA and EGTA, however, were inconclusive. For EDTA treatments, the intensity of two protein bands (38 and 25 kDa) was significantly different from that of deionized water controls. The intensity of other EDTA treatment proteins and all EGTA treatment proteins did not differ significantly from controls. Therefore we are left to conclude either that barnacle cement enzymes are not Ca^{2+} dependent or that our assay was unable detect Ca^{2+} dependence. We suspect the latter, and predict that serine protease(s) and transglutaminase are binding Ca^{2+} rapidly, prior to the time frame relevant to our polymerization assays (i.e. Ca^{2+} was already bound to the enzymes by the time EDTA or EGTA was added).

It is noteworthy that differences in protein band intensity were observed for certain EDTA treatment proteins but were not observed for EGTA treatment proteins. Whilst EGTA preferentially binds Ca^{2+}, EDTA is less specific and can bind other divalent cations (Marshak et al., 1996). This may indicate that binding of cations besides Ca^{2+} (Mn^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}) plays a role in barnacle cement polymerization. Dougherty (Dougherty, 1996; Dougherty, 1997) identified a zinc metalloprotease in *C. fragilis* cement, and showed that EDTA and orthophenanthroline decreased protease activity. Oxidizing metals may also be involved in cement polymerization through non-enzymatic means. Adhesive cross-linking *via* metal binding of adhesive proteins has been shown in multiple marine glues including that of marine mussels and gastropod molluscs (Monahan and Wilker, 2004; Sever et al., 2004; Werneke et al., 2007). Binding of cations by EDTA would decrease the formation of metal-mediated cross-links.

Conclusions

The application of evolutionary concepts and a multidisciplinary approach has helped to elucidate barnacle cement polymerization. The data presented here provide evidence that barnacle cement polymerization and blood clotting occur by similar enzymatic mechanisms. Furthermore, the presence of biochemically similar proteins in the two systems suggests that these processes may be derived from common ancestral elements. Thus, barnacle growth appears to be a specialized form of wound healing. The presence of hemocytes and proteins previously identified in hemolymph [i.e. SIPC (Dreanno et al., 2006)] in barnacle cement leads us to believe that barnacle hemolymph functions as a cement (Dickinson, 2008).

In our wound healing model for barnacle cement polymerization, cement is released during growth and repair, and contains structural precursors, inactive trypsin and inactive transglutaminase (contained within hemocytes). Trypsin-like serine proteases activate cement structural and proteolytic precursors. Activation of cement structural proteins maximizes bonding interactions, facilitating their assembly and rearrangement with the surface. Covalent cross-linking, brought about by hemocyte-released transglutaminase, reinforces the cement. Reorganization and covalent cross-linking of activated structural proteins result in an insoluble mesh of interwoven fibrous proteins. Throughout the cement polymerization process, arginine terminal peptides generated by protease activity are released, stimulating the settlement of barnacle cyprid larvae. The mechanisms described here may be a theme for many marine animal glues.

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