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The effects of a serine protease, Alcalase®, on the adhesives of barnacle cyprids (*Balanus amphitrite*)

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Barnacles are a persistent fouling problem in the marine environment, although their effects (eg reduced fuel efficiency, increased corrosion) can be reduced through the application of antifouling or fouling-release coatings to marine structures. However, the developments of fouling-resistant coatings that are cost-effective and that are not deleterious to the marine environment are continually being sought. The incorporation of proteolytic enzymes into coatings has been suggested as one potential option. In this study, the efficacy of a commercially available serine endopeptidase, Alcalase® as an antifoulant is assessed and its mode of action on barnacle cypris larvae investigated. *In situ* atomic force microscopy (AFM) of barnacle cyprid adhesives during exposure to Alcalase supported the hypothesis that Alcalase reduces the effectiveness of the cyprid adhesives, rather than deterring the organisms from settling. Quantitative behavioural tracking of cyprids, using Ethovision™ 3.1, further supported this observation. Alcalase removed cyprid 'footprint' deposits from glass surfaces within 26 min, but cyprid permanent cement became resistant to attack by Alcalase within 15 h of expression, acquiring a crystalline appearance in its cured state. It is concluded that Alcalase has antifouling potential on the basis of its effects on cyprid footprints, un-cured permanent cement and its non-toxic mode of action, providing that it can be successfully incorporated into a coating.

Keywords: barnacle; cyprid; biofouling; AFM; protease; enzyme

Introduction

Protease enzymes have many commercial uses, ranging from their incorporation in common detergents to industrial processes. Significant effort has been directed towards immobilisation of enzymes in polydimethly siloxane (PDMS) films (Avnir et al. 1994; Wu et al. 1994; Gill et al. 1999; Gill and Ballesteros 2000; Kim et al. 2000) since, in that form, they can be used as reactive surfaces or catalysts in industrial applications. Although the controlled release of biologically active compounds from coatings remains a challenge, the incorporation of such catalysts into synthetic coatings is becoming routine in industry. Production of coating films containing delicate macromolecules must currently involve 'sol – gel' processes and low temperature hydrolysis of the necessary biological monomers (Avnir et al. 1994; Dave et al. 1994). These methods have become commonplace for coatings containing sensitive biological materials since they tend to preserve large, fragile molecules such as enzymes more effectively than the traditional melting of silica (Kim et al. 2000) in silica-based film production. Cost-effective, enzyme-based industrial coatings have not yet reached the market place in spite of their demonstrated efficacy as cleaning agents against strongly adhered protein films (eg Turner et al. 2005). It has been suggested (Pettitt et al. 2004; Olsen et al. 2007; Leroy et al. 2008) that serine proteases could be included in novel fouling-resistant coatings to prevent the attachment of marine fouling organisms, such as *B. amphitrite* (Clare and Høeg 2008) through hydrolysis of their adhesive proteins on contact with the active surface. The efficacy of enzymes in this role has already been alluded to in the antifouling strategy of pilot whales (Baum et al. 2002).

Effective fouling resistant coatings, whether they are based on antifouling (eg Evans et al. 2000; Omae 2003; Jelic-Mrcelic et al. 2006; Statz et al. 2006) or fouling release technology (eg Adkins et al. 1996; Stein et al. 2003; Chaudhury et al. 2005; Kavanagh et al. 2005), are high value commodities particularly in view of the international ban on the use of TBT (tributyltin) based paints (Evans et al. 2000). The economic costs associated with marine fouling run into billions of dollars annually (Yebra et al. 2004). Barnacles are particularly pervasive foulers (Otani et al. 2007),

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causing a significant increase in drag (Schultz 2007) due to their relatively large size, hard-calcareous form (Anderson 1994) and gregarious nature (Crisp and Meadows 1962; Clare and Matsumura 2000). The adhesion strength (eg Kavanagh et al. 2005; Wendt et al. 2006) of large adult barnacles has historically been the focus of antifouling studies on fouling-release coatings. However, more attention is now being given to the settlement stages of barnacles, ie the planktonic larvae.

The settlement stage of barnacles is a cypris larva (cyprid) that actively explores immersed surfaces using a method of reversible attachment known as temporary adhesion, which is poorly understood. Temporary adhesion is facilitated, at least in part (Yule and Walker 1984, 1985; Clare et al. 1994; Dreanno et al. 2006a), by the secretion of a glycoprotein material from glands within specialised antennular structures that cyprids use for 'walking' (Nott and Foster 1969). This material has often been referred to in the literature as the cyprid temporary adhesive. During exploration, cyprids deposit 'footprints' of glycoprotein that act as a conspecific settlement cue for subsequently exploring larvae (Yule and Walker 1985; Matsumura et al. 1998; Dreanno et al. 2006a, 2006b). Once a suitable settlement location has been selected, cyprids use a second, discrete adhesive for permanent attachment (Walker 1981; Phang et al. 2006). This permanent adhesive, or cement, is derived from a pair of specialised glands within the body of the cyprid where it is stored in secretory granules. Following neurotransmitter stimulation (Okano et al. 1996), two cement components are released via exocytosis through the antennules (Nott and Foster 1969). The precursors cure when mixed (Walker 1971; Ödling et al. 2006; Phang et al. 2006) into a globular disc embedding the anntenules of the cyprid and attaching it permanently to the selected surface (Walker 1981). Metamorphosis into a juvenile occurs within hours in *B. amphitrite* and the adult adhesive develops some time thereafter.

Pettitt et al. (2004) screened twenty-five enzymes for antifouling activity including lipases, cellulases, glucoamylases and multi-enzyme complexes. Of these, the serine-proteases (specifically the enzyme preparation, Alcalase®) were found to be most effective in preventing settlement of zoospores of the green alga *Ulva* and cyprids of *B. amphitrite*. Alcalase (Novozymes, Denmark) is a commercial preparation of the serine endopeptidase Subtilisin an enzyme initially obtained from *Bacillus subtilis*. The Alcalase formulation is marketed as offering, ''increased savings due to less fouling and the need to clean equipment''. It is this putative antifouling action that is addressed in the present study.

Unpublished data (N. Aldred, S. L. Conlan, personal observations) suggested that the reduction of barnacle cyprid settlement in response to Alcalase was due, at least in part, to the effects of the enzyme on the adhesives of barnacle cyprids. Serine proteases catalyse hydrolysis of the covalent peptide bonds between amino acids within proteins (Hunkapiller et al. 1975). Although modes of action vary, these enzymes generally facilitate proteolytic cleavage through nucleophilic attack of the targeted peptide bond by a serine or aligned side chains of serine, histidine and aspartate, which are common to most serine proteases (Voet and Voet 2004; for an example see Olivieri et al. 2002). It appears from Pettitt et al. (2004), and from personal observation, that Alcalase directly affects the ability of cyprids to attach rather than reduce their willingness to attach. No direct evidence for this was presented, however, Pettitt et al. (2004) demonstrated a weakening effect of Alcalase on the permanent adhesive of cyprids.

The present study examines the hypothesis that the principal mode of action of Alcalase in its role as an antifoulant is its ability to attack the adhesive proteins of barnacle cyprids. This is explored using a combination of settlement assays, behavioural assays and direct observation of the cyprid temporary and permanent adhesives. Atomic force microscopy (AFM) was used to study cyprid adhesives in response to proteolytic attack. AFM measures the nano-scale properties of natural bioadhesive materials and adhesive interfaces in native conditions (Phang et al. 2007), ie hydrated in a saline solution, making it well suited to studies of this type. AFM has been used previously in similar studies, for example, to observe the enzymatic degradation of spin-coated poly(trimethylene carbonate) films by lipase solutions from *Thermomyces lanuginosus* (Zhang et al. 2005). Zhang et al. (2005) observed a reduction in film thickness using AFM and also demonstrated that the enzyme significantly affected film roughness over the duration of their study. The AFM results presented here demonstrate the nanostructure of both types of barnacle cyprid adhesive and report the modulation of adhesive forces and corresponding changes in morphology for 'footprint' deposits exposed to Alcalase.

Materials and methods

Cyprid culture

B. amphitrite cyprids were cultured as described by Hellio et al. (2004).

Preparation of Alcalase solutions

The Alcalase solution (4.4% protein, specific activity: 0.057 μmoles glycine μg protein^{-1} h^{-1}; Pettitt et al.

2004) was provided by Novozymes and was stored at 6°C. This starting solution was diluted 1:400 (equivalent to 100 μg ml^{-1} pure Subtilisin) or 1:800 in artificial seawater (ASW) for use in assays. Alcalase retained 90% of its original activity after 48 h at 28°C, although all assays presented here were completed within 24 h. Total protein content was determined by the Bradford method (Bradford 1976), although the estimated protein content of the stock solution differed considerably from the quoted concentration. This discrepancy was probably due to the lack of an appropriate control solution (ie the carrier solution with no enzyme) in the total protein assay. As a result, all enzyme solutions are referred to as dilutions of the stock. Heat denatured Alcalase was produced by boiling 500 μl aliquots of the stock enzyme solution in a water bath for 1 h, followed by cooling to 4°C and 1 h centrifugation at 8000*g*. The supernatant was removed and diluted for assays as described above.

Cyprid settlement and tracking assays

Behavioural tracking of cyprids using Noldus Ethovision 3.1 followed the protocol of Marechal et al. (2004). The parameters used in analysis are defined in Table 1.

The effects of Alcalase on the settlement-stimulating action of cyprid footprints cue were ascertained using settlement assays. Nitrocellulose membrane (0.45 μm pore size, Advantec MFS inc., USA) was fixed to both sides of acid-washed (10% v/v nitric acid) microscope slides (Fisherbrand, Fisher Scientific) using high modulus, aquarium-grade silicone sealant. Slides prepared in this way were used in sets of two. One side of each slide was conditioned with cyprid footprints by allowing ~100 cyprids to explore over the surface for 6 h in the dark at 28°C. The time period varied slightly between replicate experiments since cyprids needed to be removed from the nitrocellulose surfaces as soon as they were likely to begin settlement. Following footprint deposition, one slide of each pair was exposed to a 1:400 dilution of Alcalase for 1 h with gentle agitation, followed by a brief rinse with distilled water. The other slide of the pair was treated in the same way but using ASW (Tropic Marin™).

Slides were arranged vertically using plastic-coated steel wire in polypropylene Hi-Pack (As One Corp., Japan) containers, a surface that inhibits cyprid settlement when new. Two hundred cyprids were introduced into each Hi-Pack container in 300 ml ASW and incubated for 24 h in the dark at 28°C. Three replicate containers were used. Mean numbers of settled cyprids on: (a) footprint-treated (b) blank (c) Alcalase-treated blank and (d) Alcalase-treated footprints were calculated and compared with a control distribution using χ^2 analysis.

For footprint enumeration studies, cyprids were allowed to explore a 10 × 5 cm^2 piece of 0.45 μm pore size nitrocellulose membrane, adhered to the base of a Hi-Pack container using conductive carbon tape. Two hundred cyprids were used in 100 ml ASW. These containers were incubated for 16 h at 22°C in the dark. Following incubation, the membrane was removed and rinsed for 1 h in either nano-pure water, 1:400 Alcalase or heat denatured 1:400 Alcalase. The membrane was then immunoblotted following the protocol of Matsumura et al. (1998). Briefly, the membrane was rinsed in tris-buffered saline (TBS), immersed in blocking buffer (3% gelatine in TBS) and incubated with a 1% solution of the antibody to the 76 kDa subunit of *B. amphitrite* settlement-inducing protein complex (SIPC). Incubation with a secondary goat anti-mouse antibody (alkaline phosphatase conjugate) was followed by staining with a BCIP/NBT solution until footprints became visible. The membrane was allowed to dry out before footprint enumeration, which greatly enhanced the contrast of the stained footprints.

Table 1. The definitions of the parameters used to compare the behaviour of cyprids in different experimental treatments.

Parameter	Unit	Definition
Total distance moved	cm	Cumulative distance travelled by a cyprid in 5 min
Mean meander	degrees cm^{-1}	Sinuosity of the track – deviation from linear movement
Mean velocity	cm s^{-1}	Mean directional speed over 5 min
Total turn angle	degrees	Cumulative directional change taken 12 times s^{-1}

Collection of cyprid permanent cement

Three-day-old cyprids (*B. amphitrite*) were settled in 1 ml beads of 33 parts per thousand (ppt) ASW onto acetone-washed glass microscope coverslips (2 × 2 cm^2). Typically, they would begin to settle in numbers after ~8 h at 28°C (Phang et al. 2006) and permanent cement was then expressed over the course of a few minutes (Figure 1). The adhesive was allowed to cure for 2 h before the body of the cyprid was excised using fine tungsten needles and the remaining cement mass, with embedded antennules, was stored at 4°C in ASW during delivery from Newcastle to Twente. In Twente, the cement samples were loaded into the AFM 'wet-cell' and probed in ASW. Imaging

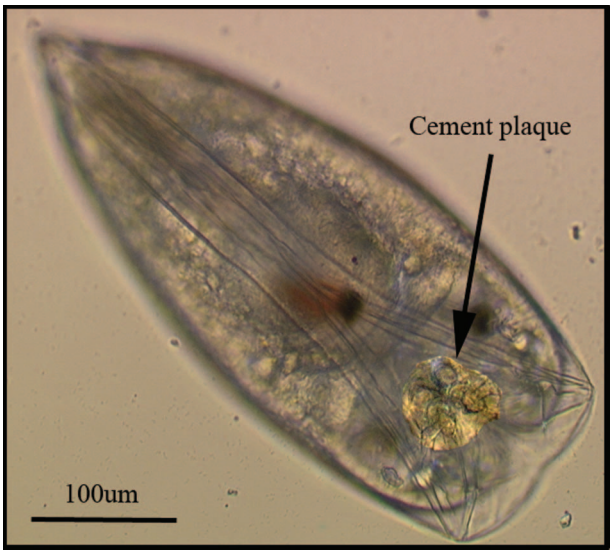

Figure 1. A settled *B. amphitrite* cyprid viewed from beneath. Note that the cement plaque with embedded antennules has been artificially accentuated.

and force measurements were conducted for at least 1 h before addition of Alcalase. After Alcalase had been introduced, AFM probing continued for up to 5 h.

Atomic force microscopy

AFM measurements were carried out using a Dimension D3100 atomic force microscope equipped with NanoScope IVa controller and a hybrid scanner (H-153) with x-, y- and z- feedbacks from Veeco (Veeco/Digital Instruments (DI), Santa Barbara, CA). Triangular-shaped silicon nitride cantilevers (Veeco/Digital Instruments (DI), Santa Barbara, CA) were used throughout the study and cantilever spring constants were calibrated using the thermal noise method. The cantilever used for acquisition of the present results had a spring constant range from 48 to 54 pN nm^{-1}. For experiments, cyprids were stored, prior to use, in 33 ppt ASW and were then deposited onto prepared silanized glass surfaces by micro-pipette. Amine-functionalized glass samples Walker 1992; (Phang et al. 2008) were adhered with carbon tape in polystyrene Petri dishes prior to experimentation. Typically, cyprids would attach and begin exploration when stimulated by small water currents. Explored areas of the glass were marked on the base of the coverslips and cyprids were then removed from the Petri dishes. Surfaces were flushed with filtered ASW to minimize surface contamination. Petri dishes were transferred to the AFM and the search for footprints was focused on the marked regions. Once footprints were located, the ASW was replaced with a 1:400 Alcalase or heat denatured 1:400 Alcalase solution. Images were taken in contact mode at 1 Hz scan rate. Nanoscope software v6.13b25 was used to transform the raw data to force-separation curves. Pull-off events occurred on these curves whenever individual proteins/protein fibrils adhered to the AFM tip on its retraction cycle. When the protein chain reached maximum extension or, alternatively, when the energy stored by extension of the protein exceeded the energy in the adhesive interaction between the tip and the protein, sharp 'pull-off' events manifested on the force-separation curve. The location of these events on the force-separation curve allowed estimation of the adhesive force between tip and substratum protein. The data (pull-off force and pull-off length) obtained from the corresponding peaks of the force-separation curves were plotted in a time-lapse fashion to demonstrate the effects of Alcalase.

Results

Settlement and behaviour of cyprids

Previous work in the authors' laboratory (Pettitt et al. 2004) demonstrated a significant reduction of settlement when cyprids were exposed to concentrations of Alcalase between 1:400 and 1:30,000 of the original stock solution. The concentration at which Alcalase was found to be acutely toxic to cyprids of *B. amphitrite* was in excess of 1:400. Pettitt et al. (2004) did not, however, determine the effects of Alcalase on the pre-settlement behaviour of cyprids. Remote video tracking of day 3 cyprids in 1:800 and 1:400 dilutions of Alcalase demonstrated no significant differences in any behavioural parameter (see Materials and Methods) between cyprids in Alcalase and those in ASW or heat-denatured Alcalase (Figure 2). Exposure to non-toxic concentrations of Alcalase, which elicited significantly reduced settlement, did not, therefore, alter the general behaviour of *B. amphitrite* cyprids when measured in this way.

Temporary adhesion of cyprids

Footprints were enumerated on nitrocellulose membrane that had been explored by cyprids and subsequently washed with either 1:400 Alcalase, heat denatured Alcalase or ASW. No significant reduction in footprint density was detected after treatment with ASW (mean density = 9.1 $\pm$ 1.2 SE footprints cm^{-2} ($n = 30$)) and heat denatured Alcalase (mean density 11.6 $\pm$ 1.4 SE footprints cm^{-2} [$n = 30$]). However, no footprints were observed on membrane that had been treated with active 1:400 Alcalase.

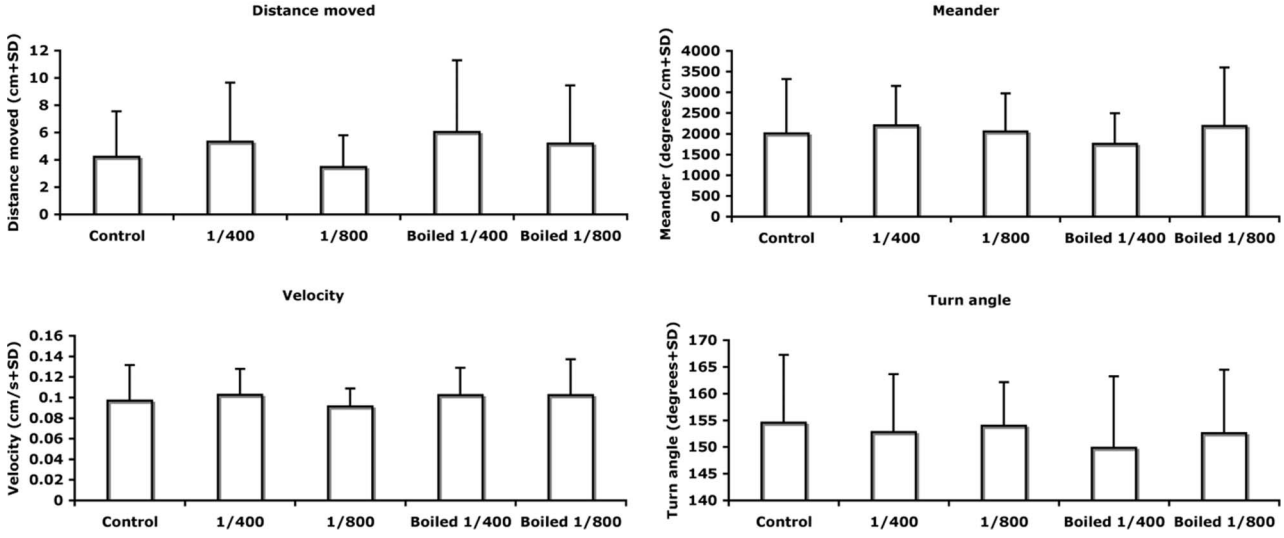

Figure 2. Behavioural data acquired by remote video tracking of *B. amphitrite* cyprids in Alcalase solutions. 1:400 and 1:800 dilutions of Alcalase did not significantly affect the overall behaviour of cyprids.

To determine whether enzymatic removal of footprints negated their inductive (pheromonal) effect on settlement of cyprids, a choice assay was performed between clean (control) and previously explored (footprint-treated) surfaces that had been exposed either to Alcalase or ASW. When 3-day-old cyprids were used, there was a significant bias towards settlement on footprint-treated surfaces that had not been exposed to Alcalase ($\chi^2 = 10.89$, $P = 0.012$). Settlement on the other test surfaces appeared equal (Figure 3).

AFM provided direct evidence for enzymatic proteolysis of cyprid footprints. Individual footprints could be scanned in ASW in contact mode for hours consecutively and their nanomechanical properties investigated both before and after the introduction of a 1:400 Alcalase solution. The technical difficulty of locating and scanning footprint deposits with AFM precluded the desired level of replication. Nevertheless, on three occasions footprints were noted to be entirely removed from a glass substratum 20–25 min after the introduction of 1:400 Alcalase (Figure 4). No morphological changes were detected in footprints exposed to either ASW or heat denatured Alcalase for the duration of the experiments. AFM observation of footprints on glass suggested that in the absence of an external influence, footprints would persist unmodified for days in either ASW or heat denatured Alcalase.

Figure 5 shows the effect of Alcalase on the morphology of the cyprid footprint in Figure 4. Before addition of Alcalase, *B. amphitrite* footprints deposited on glass were of the order of 30 μm diameter and 15 nm thick. The thickness of the footprint deposits

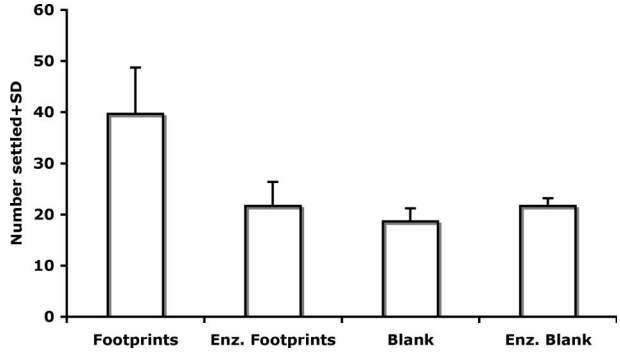

Figure 3. The number of *B. amphitrite* day 3 cyprids settled in a 24-h choice assay. Cyprids were allowed a choice between nitrocellulose surfaces with previously deposited footprints (''Footprints''), blank control nitrocellulose surfaces (''Blank'') and those two types of surface that had also been exposed, prior to the assay, to 1:400 Alcalase (viz. ''Enz. Footprints'' and ''Enz. Blank'', respectively).

varies depending on surface chemistry/hydrophobicity (Phang et al. 2008). From Figures 4 and 5 it is clear that footprint deposits were 'doughnut' shaped, with a central circular area of ~10 μm diameter that contained very little glycoprotein secretion (see Walker 1992; Phang et al. 2008). The surface structure of the footprints suggested that the proteinaceous material was drawn into fibrils on removal of the antennular disc from the surface.

Figure 6 presents data acquired from one cyprid footprint during enzymatic proteolysis. The pull-off force of individual protein fibrils (Figure 6A) that had adhered to the cantilever tip and the length that fibrils reached before detaching from the tip (Figure 6B) were

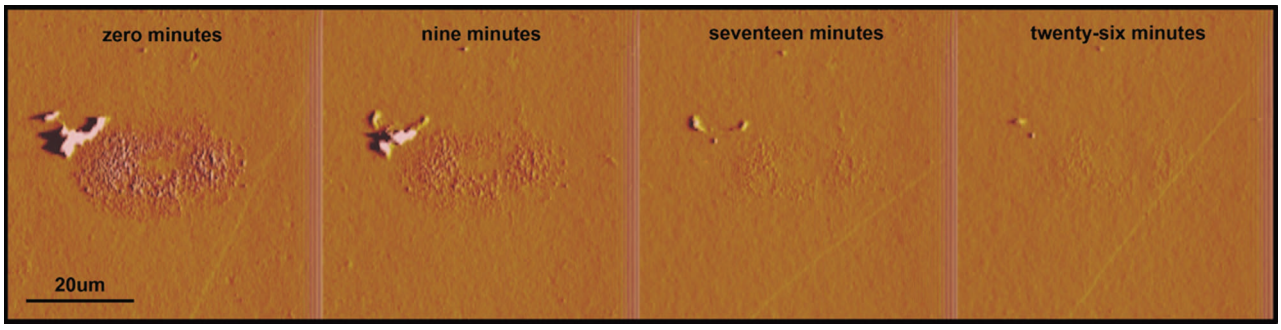

Figure 4. The proteolytic removal of a *B. amphitrite* footprint from a glass surface over the course of 26 min. Images are AFM phase traces.

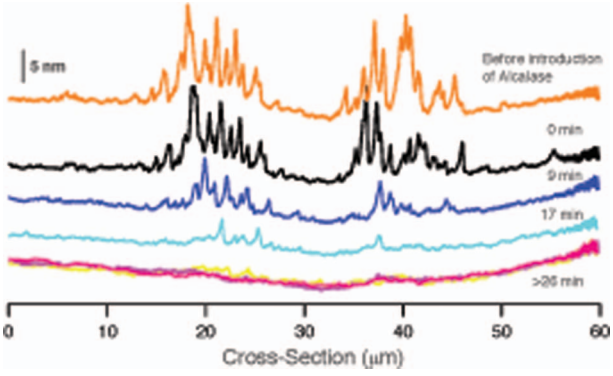

Figure 5. AFM height profiles of the footprint in Figure 6 recorded over the course of 26 min.

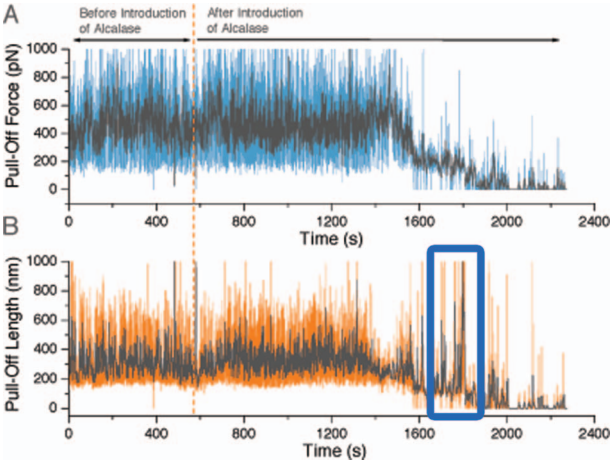

Figure 6. Data from AFM analysis of *B. amphitrite* footprints before and during exposure to Alcalase. (A) a plot of pull-off force between the AFM tip and the footprint over time. (B) a plot of pull-off length – the distance from the footprint over which proteins remained attached to the AFM tip, over time. The dark line in both curves is a 5-point average and the blue rectangle highlights a series of unusually high pull-off length events around 1800 s (30 min).

highly variable, but constant over the duration of testing in ASW. Although only 10 min of ASW control data are presented in Figure 6, this control period lasted, in reality, for several hours. Only after the introduction of Alcalase was there any change in either of these two parameters. Even though Figures 4 and 6 represent different footprints, the time frame in which the enzyme solution acts was similar for both. Thirteen minutes after the addition of Alcalase (at 1450 s in Figure 6A and 6B), the pull-off force began to decrease with a concurrent reduction in variability of the pull-off force distribution (Figure 6A). The variability of pull-off length data also decreased. There was, however, a series of unusually high pull-off length events throughout the time course of the experiment that suggested some fibrils were still being drawn out to considerable lengths until the footprint had been removed completely (highlighted blue in Figure 6B). After a 16-min exposure to Alcalase, only a trace of the footprint shown Figure 6 remained on the substratum with few pull-off events evident from 2000 s onwards in Figure 6A and 6B.

Alcalase, introduced at 564 s, reduced the pull-off force (Figure 6) in a step-wise manner. The pull-off force dropped from 340 pN (between 600 and 1400 s) to a plateau at 150 pN before dropping again to virtually zero, the native force of the glass substratum.

Permanent adhesion of cyprids

After 48 h in ASW, the permanent cement of *B. amphitrite* appeared to be totally resistant to Alcalase. The AFM tip did not pick up any proteins at the surface of the cement plaque, either before or after addition of the enzyme. Adhesion of surface proteins to the tip was common in uncured cement (Phang et al. 2006, 2007). Figure 7 shows the structure of cured permanent cement from *B. amphitrite* prior to Alcalase exposure and this surface structure remained

unchanged after a 3 h Alcalase exposure. High resolution scans of the permanent cement were possible in this cured state, in contrast to the deposited cement described in Phang et al. (2006).

In contrast, uncured cement (~1 h old) was highly susceptible to Alcalase (Figure 8), as reported by Pettitt et al. (2004), and was visibly thinned over the course of the 5 h exposure. If the cyprid was attached to the cement plaque during this period then its movements invariably caused it to dislodge from the surface. Only when the cyprid had been manually removed did the adhesive plaque remain attached to the surface on exposure to Alcalase. This thinning of the adhesive plaque did not occur if the cement was first stained with a fixing protein dye such as Coomassie Brilliant Blue (acetic acid and methanol preparation).

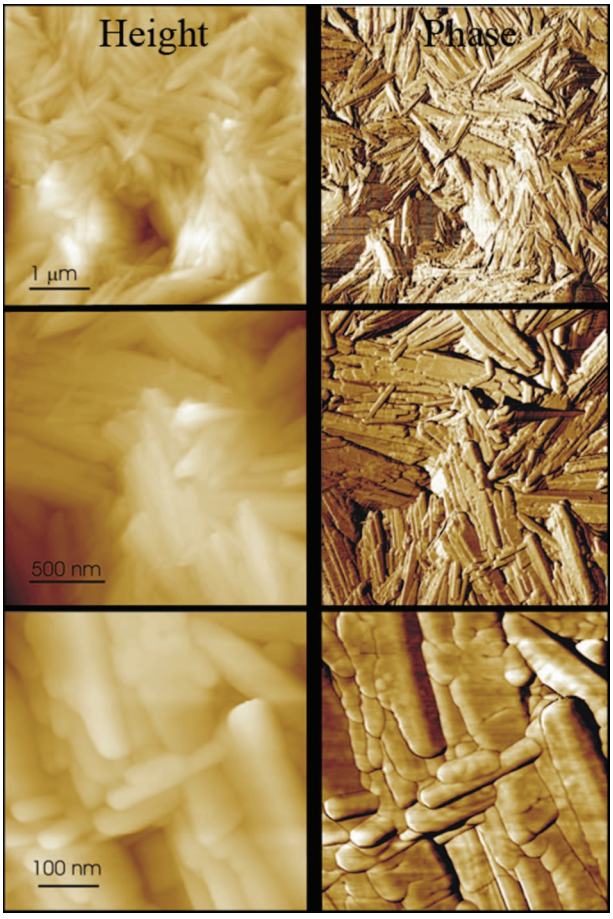

Figure 7. AFM contact mode images of cured (3-day-old) and hydrated *B. amphitrite* cyprid permanent cement. This cement had not been exposed to Alcalase. Cement of the same age that was exposed to Alcalase appeared identical when viewed by AFM suggesting that cured cement is resistant to Alcalase attack.

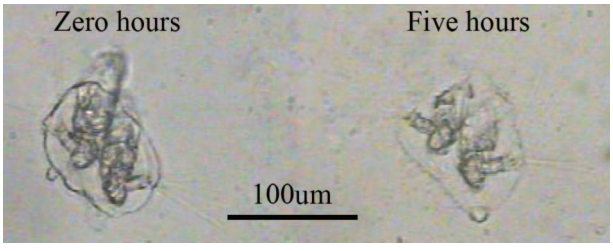

Figure 8. Cyprid permanent cement that is not fully cured visibly thinned during exposure to a 1:400 solution of Alcalase.

Discussion

The present study aimed to extend the scope of the experiments reported by Pettitt et al. (2004), by using surface topology imaging and nanomechanical probing by AFM to provide a mechanistic understanding of the non-toxic inhibitory action of Alcalase on barnacle cyprid settlement. It is important to mention that since Alcalase is a commercial preparation of Subtilisin, it is impossible to attribute the findings of this study entirely to the action of Subtilisin. There remains a possibility, albeit unlikely, that another undisclosed element of Alcalase, which can also be heat inactivated, is responsible for the present observations. Therefore further experiments using pure Subtilisin will be the next phase of study. Alcalase was used here to further illustrate and directly expand upon previous observations (Pettitt et al. 2004).

The hypothesis that modulation of cyprid settlement by the enzyme was principally driven by hydrolysis of the cyprid adhesives was supported by AFM analysis and immunostaining. It was shown that the cyprid antennular secretion (in the form of footprints), which is critical to surface exploration, did not persist for more than a few minutes when exposed to a concentrated solution of Alcalase. Although antibody staining of surfaces explored by cyprids appeared to show total removal of footprints by Alcalase, it is recognised that the enzyme could interfere with the immunostaining technique by blocking the binding sites of the 76 kDa peptide-specific antibody. The ability of Alcalase to completely remove cyprid footprints however, as demonstrated by AFM (Figure 4), suggests that the disappearance of footprints from the nitrocellulose membrane was a result of Alcalase exposure rather than a failure of the staining method. Dissolution of the cyprid's glycoproteinaceous antennular secretion would, presumably, reduce its attachment tenacity during exploration (Phang et al. 2008) and, therefore, the likelihood of their settlement (Neal and Yule 1992); even if the cyprids were still physically capable of settling. Remote video tracking suggested that an aqueous solution of

Alcalase, of a sufficiently high concentration to reduce settlement, did not affect the pre-settlement behaviour of cyprids in any measurable way. This result suggests that the inhibitory action of Alcalase on cyprid settlement is via a non-toxic mode of action, which is an important environmental and regulatory consideration (see Olsen et al. 2007).

Interestingly, the material properties of cyprid footprints did not change over the duration of exposure to ASW and the inductive properties of footprints are known to persist for days to weeks in the natural environment (Yule and Walker 1985). An implication is that the adhesive/de-adhesive ''duo-gland'' hypothesis proposed by some for temporary adhesion in starfish (Flammang et al. 1994) is unlikely to be applicable to cyprid antennule detachment. If the cyprid secreted its own enzymes to break down the antennular secretion and facilitate release, the progressive effects of those enzymes would be detected by AFM. Moreover, the glycoprotein that is thought to comprise footprints (Matsumura et al. 1998; Dreanno et al. 2006a, 2006b) had significant topography (Figure 5) after removal of the cyprid antennule from the surface, suggesting that the adhesive was still connected to the antennular disc during detachment. It was not possible to discern whether the adhesive failure was cohesive within the cyprid footprint itself, or adhesive between the footprint and the antennular disc. However, maintaining as much material on the adhesive disc as possible would reduce the necessity for costly production and would be an advantageous evolutionary trait for cyprids.

The reductions in both pull-off force and pull-off length of footprint proteins after the addition of Alcalase (Figure 6) may be directly linked. If a protein fibril was not effectively attached to the AFM tip, if it was removed by Alcalase during extension, or lysed along its length, it would predictably have a low pull-off length and force. The drawing out of footprint protein fibrils by the AFM tip (Phang et al. 2007) would have exposed more sites to Alcalase making proteolysis more likely. The longer the footprint was exposed to Alcalase the more proteolysis events would have occurred, continually reducing the mean length of protein fibrils in the deposits and also reducing the mean pull-off length. The high pull-off length events (Figure 6B) that persisted until the end of the experiment suggested that even until late in the time series some full-length protein fibrils remained.

It was not the intention of the present study to provide a mechanistic explanation for cyprid temporary adhesion. An interesting observation, however, was that cyprid footprints were 'hollow' (Figure 5) when viewed from above by AFM (Walker 1992). The authors do not propose suction to be important to cyprid attachment (cf Lindner, 1984) and furthermore, this hypothesis was disproved by Nott and Foster (1969) and Yule and Walker (1987), but this void may still play an important role in attachment or detachment. Alternatively, it could provide an uncontaminated area for surface sensation by the axial sense organ (Nott and Foster 1969) in the centre of the adhesive disc.

Pettitt et al. (2004) noted that fresh permanently attached cyprids (Figure 1) became quickly detached from their settlement surface after exposure to 1:400 Alcalase. After $\sim$15 h post-settlement, however, Pettitt et al. (2004) observed that Alcalase no longer facilitated removal and suggested that either a new, Alcalase-resistant adhesive was produced at this time, or that the cyprid cement had fully cross-linked (Walker 1971) and was therefore impervious to attack by the enzyme. Further evidence for curing of cyprid cement was provided here in that no effect of exposure to Alcalase was detectable in 48-h old cement. This result suggests that in the early post-metamorphosis stage of barnacles it is the fully cross-linked cyprid cement that mediates attachment rather than a discreet juvenile adhesive system. The cyprid cement persists, viewable from beneath the basis, throughout adulthood in barnacles (Yule and Walker 1987). In the light of previous results (Phang et al. 2006), it was something of a surprise that cement curing, as defined by Alcalase resistance, should take up to 15 h from the time of expression. (Phang et al. 2006) provided evidence that *B. amphitrite* cyprid cement cures within 70–120 mins of release to the substratum. The measurements in the first study were made, however, only on the outermost layer of cement, where curing may be catalysed by available trace metals, oxygen or water. Both observations are, therefore, valid.

The permanent cyprid cement (Figure 7) had a distinctly crystalline appearance when cured that had not been observed in the only other AFM study of this material (Phang et al. 2006). It is not known if these features were crystals in the true sense, and only close study using X-ray techniques could confirm that hypothesis. However, it is known that the cement likely self-assembles via quinone tanning from the peptide monomers kept in granular form within α and β cells in the cyprid cement glands (Walker 1971). The permanent adhesive apparatus of cyprids could, hypothetically, be similar in this regard to the silk production system of spiders, albeit simplified. Silk-spinning in spiders (Vollrath and Knight 2001) works by a mechanism of storage and secretion, from specialised glands, of a water-soluble material (the 'spinning-dope') that is added to during extrusion to form a water-resistant crystalline-matrix thread. Much of the spider's elaborate silk-spinning machinery is

involved in formation of a usable thread. This is not required by cyprids, so their mechanism for simply expressing a volume of cured crystal-matrix material need not be so complex. Requisites would be water-soluble precursors (Walker 1971), a collecting duct where curing agents could be added (Nott and Foster 1969) and the ability to express the material before it cures (Phang et al. 2006).

The cyprid cement in Figure 7 is morphologically different to the adult barnacle cement (Berglin and Gatenholm 2003; Sun et al. 2004; Nakano et al. 2007), allaying the debate surrounding whether or not the two may be the same or related. Walker (1971) first observed that material from the cyprid cement glands migrates during metamorphosis and is included in the adult cement glands, although it seems that the product of the adult adhesive machinery is quite distinct from that of its cyprid counterpart. Further, Kamino and Shizuri (1998) demonstrated that genes encoding adult cement production were not expressed in the cyprid, making any biochemical similarity in the adhesives unlikely.

The exact mechanisms by which artificial chemical fixation (shown here using Coomassie Brilliant Blue) and putative quinone cross-linking (a natural process in cyprid cement curing) invoke Alcalase resistance in cyprid permanent cement are unknown, but are probably similar in effect since artificial fixation generally occurs via covalent cross-linking of proteins. Covalent bonds formed during fixation are not the common peptide bond that Alcalase acts on and, therefore, remain unaffected by the presence of Alcalase. Quinone cross-linking is highly variable and not completely understood. Bonds formed during the tanning of insect cuticle, for example, are highly complex. Quinones of N-acylated catecholamines, such as N-acetyldopamine (NADA) or N-*b*-alanyldopamine (NBAD) are known to undergo nucleophilic addition with amino acids such as histidine, resulting in bonds that would also be resistant to Alcalase (Bittner 2006). Given the potent effects of Alcalase on barnacle cyprid adhesives, as well as on the adhesives of algae and diatoms (Pettitt et al. 2004), it would seem that proteolytic enzymes of this type have considerable antifouling potential. Now that their efficacy has been proven, it is the task of coatings manufacturers to incorporate enzymes into affordable systems that maintain their biological activity over a long operational period (at least 1 year). If this is accomplished, enzyme-based coatings could find wide application wherever underwater fouling is an issue.

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