Biochemical analyses of the cement float of the goose barnacle *Dosima fascicularis* – a preliminary study

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The goose barnacle *Dosima fascicularis* produces an excessive amount of adhesive (cement), which has a double function, being used for attachment to various substrata and also as a float (buoy). This paper focuses on the chemical composition of the cement, which has a water content of 92%. Scanning electron microscopy with EDX was used to measure the organic elements C, O and N in the foam-like cement. Vibrational spectroscopy (FTIR, Raman) provided further information about the overall secondary structure, which tended towards a β-sheet. Disulphide bonds could not be detected by Raman spectroscopy. The cystine, methionine, histidine and tryptophan contents were each below 1% in the cement. Analyses of the cement revealed a protein content of 84% and a total carbohydrate content of 1.5% in the dry cement. The amino acid composition, 1D/2D-PAGE and MS/MS sequence analysis revealed a de novo set of peptides/proteins with low homologies with other proteins such as the barnacle cement proteins, largely with an acidic pI between 3.5 and 6.0. The biochemical composition of the cement of *D. fascicularis* is similar to that of other barnacles, but it shows interesting variations.

**Keywords:** barnacle adhesive/cement; 1D/2D PAGE; FTIR/Raman spectroscopy; protein primary/secondary structure; hydrogel; carbohydrate

**Introduction**

Proteinaceous underwater adhesives are secreted for locomotion by the sea star and sea urchin (Flammang et al. 1998; Santos et al. 2009), for defence by the sea cucumber (DeMoor et al. 2003), for building protective tubes by sabellariid polychaetes (Stewart et al. 2004) and for attachment to the substratum by mussels and barnacles (Kamino et al. 1996; Waite 2002). The best characterised marine bioadhesive systems are those of the sandcastle worm *Phragmatopoma californica* (Stewart et al. 2004), the blue mussel *Mytilus edulis* (Silverman & Roberto 2007) and the acorn barnacle *Megabalanus rosa* (Kamino 2008). The adheres of the sandcastle worm (Wang et al. 2010) and mytilid mussels (Waite 2002) contain significant amounts of the amino acid L-3,4-dihydroxyphenylalanine (L-DOPA). L-DOPA can complex with metal ions and oxides (Sever et al. 2004), as well as with semimetals, such as silica, therefore allowing adherence to rocks and glass (Silverman & Roberto 2007). The holdfast system of the barnacle differs from that of mytilid mussels and sabellariid polychaetes by the lack of a detectable L-DOPA system and the slow curing process of the adhesive (Naldrett 1993; Kamino et al. 1996; Kamino 2010; Nicklisch & Waite 2012). Barnacle cement is produced by unicellular glands throughout the lifetime of the animal in accordance with its moulting cycle (Saroyan et al. 1970; Fyh & Costlow 1976; Jonker et al. 2012; Zheden et al. 2012). Two types of adhesive can be distinguished: the primary cement, produced while the animal is attached to a substratum, and the secondary cement, secreted when the animal is injured or detached (Saroyan et al. 1970, 1970; Kamino et al. 1996). The two kinds of cement are very similar in their amino acid composition (Naldrett 1993) and consist of ~90% protein (Walker 1972; Kamino et al. 1996). Usually the secondary cement is used for biochemical analyses, because it is easier to collect.

Most studies on the cement proteins of cirripedes have been conducted on acorn barnacles, especially *Megabalanus rosa*. Its cement is composed of more than 10 proteins, of which five are novel in their primary structure compared with other underwater adhesives (Kamino 2006). Each protein has a unique characteristic and it is assumed that it fulfils either a surface or bulk function in the multifunctional process of underwater attachment.

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Only a few studies exist on the biochemistry of the cement of goose or stalked barnacles. The first investigations of the biochemical composition of the cement of the buoy barnacle *Dosima (Lepas) fascicularis* (Ellis & Solander 1786) were by Barnes and Blackstock (1974, 1976). They determined the carbohydrate, lipid and amino acid composition as well as the electrophoretic properties of the cement. Walker and Youngson (1975) focused on the related goose barnacle *Lepas anatifera* and assumed that the cement of acorn and goose barnacles had a similar biochemical composition, but that it may act in a different way. Goose barnacles normally secrete a thick layer of primary cement in contrast to the thin film produced by acorn barnacles (Walker & Youngson 1975). Most pelagic goose barnacles of the family Lepadidae are, like acorn barnacles, known to be major fouling organisms. They often cover floating objects such as ships, buoys, tar pellets, plastic, driftwood, feathers and seaweed (Boëtius 1952–53; Cheng & Lewin 1976; Minchin 1996; Hinojosa et al. 2006). *Dosima fascicularis* does not usually cover large surfaces. It clusters either on organic or inorganic substrata of different size or on the cement of conspecifics. Its stalk is flexible and the plates are fairly soft. It is unique among the barnacles due to the amount and morphological structure of the cement it produces. It secretes layer by layer an excess cement it produces. It secretes layer by layer an excess amount of foam-like cement (Zheden et al. 2012) (Figure 1 and Supplementary Figure S1). [Supplementary material is available via a multimedia link on the online article webpage.] The bubbles in the foam-like cement are probably filled with CO₂, a metabolic product of the haemolymph (Zheden et al. 2012). As the animal grows, small substrata such as feathers or pieces of seaweed can be surrounded and sometimes enclosed by the enlarging cement, which then forms a float (Figure S1a, b). As a result, *D. fascicularis* can drift on the surface of the water (Boëtius 1952–53; Young 1990).

In the present study some of the biochemical components of the cement of *D. fascicularis* were investigated. The large amount of primary cement is easily accessible and therefore ideal for analysis. Although there is overall agreement on the composition of the cement of *D. fascicularis* compared with that of other barnacles, some differences are noticeable, including the acidic composition of the adhesive proteins, their structural architecture, the absence of Raman detectable disulphide bonds and the very low amounts of cystines.

**General cement analysis**

The cement was removed from the animal and any adherent substratum. Pieces of cement were washed in distilled water to remove debris and salt before freezing at −20°C or freeze-drying (Christ Alpha1–4, Christ GmbH, Osterode, Germany). To estimate the water content of the cement, four samples of three different individuals of *Dosima* were weighed before and after freeze-drying. For the protein analyses, the freeze-dried samples were powdered in a small porcelain mortar.

**Total carbohydrate analysis of the cement**

Carbohydrate analysis was performed using a kit from BioVision (Catalogue # K645–100. Milpitas, CA, USA). This is based on the phenol-sulphuric acid method and can detect most forms of carbohydrates, including simple and complex saccharides, glycans, glycoproteins and glycolipids. The experiments were performed according to the supplier’s manual. Freeze-dried cement and glucose for the standard curves (supplied in the kit) were completely dissolved in 150 μl of concentrated H₂SO₄ (98%) and incubated at 90°C for 15 min. Thereafter 30 μl of developer were added and the mixture was mixed on a shaker for 5 min. Control analysis took place without the developer. The samples were detected spectrophotometrically at 490 nm with a multimode reader Mithras LB940 (Berthold Technologies, Bad Wildbad, Germany). The total carbohydrate content of the freeze-dried material was calculated on the basis of the glucose standard curve via linear regression analysis.

**Staining methods**

The periodic acid-Schiff (PAS) method (McManus & Mowry 1960) was used to detect the presence of neutral hexose sugar units in the cement. The negative control did not contain periodic acid. The slime of the slug *Arion rufus* served as a positive control. To test for the possible presence of L-DOPA in the cement, pieces were stained according to the protocol of Arnow (1937). Samples from the tube-dwelling polychaete *Sabellaria alveolata* were used as a positive control (Becker et al. 2012).

**Scanning electron microscopy with energy dispersive X-ray microanalysis (EDX)**

The cement float was cut into pieces, washed with distilled water overnight and air-dried. The elemental composition was determined without any carbon coating on the samples using a Philips XL 20 scanning electron microscope (SEM) (Eindhoven, The Netherlands) equipped with an energy dispersive X-ray spectrometer with a lithium-drifted silicon (SiLi) detector crystal.

**Materials and methods**

*Dosima fascicularis* was collected during the summer months of 2012 and 2013 on the west coast of Denmark (56°57’37.63” N, 8°21’37.60” E) after having been washed ashore. Only primary cement was used for the analyses.
Analyses were performed using EDAX Genesis 5.11 software (Mahwah, NJ, USA). Spectra of the elements detected in the cement were collected over a time of 100 s and a ~30% dead time. Line scans with a distance of 140 μm and dot mappings (resolution 256 x 200, 22 frames) of the elements were also acquired. Operating parameters for all these analyses were: accelerating voltage 20 kV, acquisition at 15° take-off angle and a working distance of 12 mm. For high-resolution images, some samples were coated with gold using an Agar B7340 sputter coater (Stansted, Essex, UK) and examined in the SEM at an accelerating voltage of 15 kV.

**Amino acid analysis**

The amino acid composition of the cement was determined by the Institut Kuhlmann GmbH (Ludwigshafen, Germany). A small piece of the cement was washed several times with ultra pure water and then freeze-dried. Three samples were hydrolysed and diluted in buffer solution. The amino acid composition was then determined using an S443 analyser (Sykam GmbH, Fuerstenfeldbruck, Germany). After chromatographic separation of the amino acids they were post-column derivatised with ninhydrin and detected at 570 nm (prolin 440 nm). For cystine and methionine analysis the samples were treated with performic acid for 15 h and then hydrolysed for 24 h with 6 N HCl at 120°C. The amino acid tryptophan was analysed after alkaline hydrolysis for 24 h with 5.6 N NaOH at 110°C. All other amino acids were hydrolysed under acidic conditions with 6 N HCl at 120°C for 24 h.

**Solubilisation experiments**

The freeze-dried cement was solubilised in thiourea/urea lysis buffer (2 M thiourea, 7 M urea, 2% (w/v) CHAPS), 2% (w/v) and alternately 5% (w/v) dithiothreitol (DTT)) by incubation at 37°C overnight with stirring (Rabilloud 1998). Afterwars the samples were centrifuged at 21,000 x g for 15 min. The supernatant was removed and stored (−20°C). The remaining pellets were dissolved in SDS sample solubilisation buffer (1% SDS, 100 mM Tris-HCl pH 9.5) and incubated at 37°C for 3 h with stirring. After centrifugation the final soluble fraction was stored (−20°C).

**Electrophoretic analyses**

Samples for one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) were incubated at 95°C for 15 min in Laemmli sample buffer (Bio-Rad, Munich, Germany). The samples were run on a 1D precast 10–20% polyacrylamide ready gel (Bio-Rad) with two different amounts of protein (10 and 20 μg). Precision Plus Protein Dual Colour Standards (Bio-Rad) were used as markers. Gels were run at 100 V, 50 mA for 90 min in SDS PAGE running buffer 1x (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS at pH 8.3). The proteins were visualised by staining with Page Blue™, Protein Staining Solution (Thermo Fisher Scientific Inc., Waltham, MA, USA). For the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), the final soluble fraction was precipitated with 20% trichloroacetic acid (TCA) and 0.2% DTT in ice-cold acetone. IEF rehydration solution (2 M thiourea, 6 M urea, 1% (w/v) CHAPS, 1% (w/v) DTT, 0.5% (w/v) IPG buffer pH 3–4) was added to the fractions and incubated at 37°C for 20 min with stirring followed by consecutive centrifugation at 21,000 x g for 5 min. The protein solution (−150 μg of protein) was applied to a ReadyStrip™ IPG Strip (pH 3–10, 11 cm) (Bio-Rad). Isoelectric focusing (IEF) was performed with an Etan IPGphor3 unit (GE Healthcare, Sweden) for 13,000 Vh at 50 μA strip−1. The strips were equilibrated in equilibration buffer 1 (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% (w/v) DTT) and equilibration buffer 2 (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 4% (w/v) iodoacetamide) with gentle agitation for 15 min each. 2D-PAGE for each strip was performed at 200 V, 50 mA for 50 min in precast 10–20% polyacrylamide ready gels for IPG strips (Bio-Rad). Proteins were visualised by overnight staining in Page Blue™ and destained in distilled water (Grunwald et al. 2007).

**Sequence analysis**

Gel slices from 1D-PAGE gels of protein bands at a size of 85.2, 68.4, 63.5 and 60.5 kDa were digested by trypsin/chymotrypsin according to the company’s protein digestion instructions for In-Gel samples (protocol and performance by Proteome Factory AG, Berlin, Germany). The acidified peptides were applied to nanoLC-ESI-MS (LTQ-FT, Thermo Finnigan) analyses using a 35 min nanoLC gradient (Agilent 1,100 nanoLC system) with solvent A (0.1% formic acid, 5% acetonitrile, 94.9% ddH2O) and solvent B (0.1% formic acid, 99.9% acetonitrile). The mass accuracy was better than 5 ppm for MS data and ± 0.5 Da for MS/MS data. MS/ MS was subjected to de novo sequencing. The induced modification propionamide adducts (at cysteine) and common sample handling modifications were allowed during the MS/MS data search. To minimise false positive hits, common laboratory proteins (ie trypsin, keratin) were taken into account and their peptide signals were disregarded. Each candidate sequence was used for a BLAST alignment search to further exclude contaminants.

The de novo sequencing of the MS/MS spectra was performed by using the PEAKS® program (Bioinformatics Solutions Inc., Waterloo, ON, Canada). This software
computed the best peptide sequences based on the fragment ions collected from the peaks in the MS/MS spectrum (Zhang et al. 2012). The SPIDER program (Han et al. 2005) was used in conjunction with the PEAKS tool to perform database searches for the MS/MS generated sequence tags. The SPIDER tool is able to cope with common sequence substitutions such as I/L, N/GG, SAT/TAS in the de novo generated sequence tags. The ALC score was used as selection parameter for the MS/MS sequence tags for the resulting Dosima peptide sequence (see Table 2); only the sequences with the highest score are given here. (De novo sequencing by MS/MS is less reliable than chemical methods such as the EDMAN degradation and therefore makes scoring of the tags necessary. The selection parameter for the candidates was their ALC score for the given sequence and only de novo peptide sequences with the very highest score were given.)

The identified peptide sequences were additionally analysed by a NCBI blastp search in non-redundant database. The blastp search algorithm automatically adjusted the parameter to find optimal matches to short peptides. The search was made first without limitations in the taxa settings, and second only with the search option barnacles (taxid:6,676). The first two results of each peptide were displayed and a selection of other identified sequences is shown in Table S1.

**FTIR and Raman spectroscopy**

Protein samples were measured (a) as freeze-dried powder without any further procedure and (b) after dissolution in common D$_2$O (Sigma Aldrich, Munich, Germany). For D$_2$O exchange a fresh piece of cement was washed five times with ultra pure water (0.05 $\mu$S cm$^{-1}$) for 1 h. After this initial washing step, the cement piece was immersed in 2 ml of D$_2$O for 7 days (the D$_2$O solution was changed daily).

For interpretation of the results normal peak picking was used. The Fourier transform infrared (FTIR) measurements were performed on a Bruker Equinox 55 instrument (Bruker Optics GmbH, Ettlingen, Germany) with a Harrick Golden Gate ATR inlet (diamond crystal, one reflection). Measurements were taken with a resolution of 4 cm$^{-1}$ and 32 scans. Background measurements were taken against air. The FT-Raman measurements were performed on a Bruker Vertex 80 with RAM II module (Bruker Optics). A 1,064 nm Nd-Yg laser was used. Measurements were taken with 250 scans at a resolution of 4 cm$^{-1}$.

The spectra and images were evaluated using the Bruker software package OPUS 6.5 and the CONFOCHECK system (Bruker Optics), which is a dedicated FTIR system for the investigation of proteins. Identified peaks were aligned manually and plotted on the spectra.

**Results**

**SEM with EDX**

The excess amount of foam-like cement produced by *D. fascicularis* (Figure 1a, Figure S1) consisted of concentrically arranged layers with gas-filled bubbles of different sizes (Figure 1b, c). Near the surface of the cement, the layers were closer packed, giving the appearance of a rind containing small bubbles (Figure 1b, c).

EDX analyses gave a rough overview of the elemental composition of the primary cement. The elements carbon (C), oxygen (O) and nitrogen (N) had the highest counts in the spectrum, followed by sulphur (S) and magnesium (Mg). Calcium (Ca), phosphorus (P) and potassium (K) were present only in smaller quantities (Figure 2a). EDX line scan spectra and dot mappings of the elements showed that C, O and N were distributed evenly throughout the cement (Figure 2b, c). S, Mg, Ca, P and K seen in the spectra were not detected by line scans and dot mappings, supposedly because the amounts of these elements were below the detection limit of the system.

**Water, carbohydrate and amino acid analysis**

The cement had a high water content of 92% ± 2.7 (mean ± SD; n = 4), estimated by comparing the fresh and freeze-dried samples. The cement itself had the consistency of a stable gel, which could not be broken down by the fingers. The dried cement was found to contain 1.5% ± 0.08 (mean ± SD; n = 4) carbohydrates, as was confirmed by PAS staining (Figure S2a).

The amino acid analysis showed that the total protein content of the cement was at least 84%. Eighteen amino acids were identified by hydrolysis (Table 1). The amino acids AsX (asparagine or aspartic acid) and GIX (glutamine or glutamic acid) were most prominent, each at about 10% (Table 1). The methionine, histidine, cystine and tryptophane amino acids were only found in small quantities (<1%). The proteins contained slightly more polar (51%) than non-polar (49%) amino acids. High amounts of amino acids with hydrophobic side chains (about 43%) and only small amounts of basic amino acids (about 11%) were detected in the cement.

In summary, the cement was composed of (w/w) 92% water, 6.7% proteins (84% of dry cement), 0.12% carbohydrates (1.5% of dry cement) and 1.2% undefined substances. Part of the latter could be salts from the seawater or lipids.

**Solubility and electrophoretic properties**

The cement could not be fully solubilised with a thiourea/urea lysis buffer and some unresolved debris remained. At least 10 proteins were identified using 1D- and 2D-PAGE stained with coomassie dye PageBlue™.
Their molecular mass ranged from 47.4 to 205.0 kDa (Figure 3a). The apparent molecular masses of the prominent proteins were 60.5, 63.5, 68.4, 85.2, 111.4, 149.3 and 205.0 kDa. The 2D-PAGE showed three additional protein bands the molecular mass of which could not be confidently determined due to the lack of marker bands above 250.0 kDa. All the detected proteins were acidic (or neutral) with an isoelectric point (pI) ranging from 3.5 to 6.0 (Figure 3b). The prominent proteins in the 2D gel had molecular masses of 47.4, 60.5, 63.5, 68.4, 135.4, 149.3 and 205.0 kDa. Following the nomenclature adopted by Kamino et al. (2000) the *D. fascicularis* cement proteins were named according to their molecular masses (Dfcp-60, -63, -68 and -85).

### Sequence analysis

The sequence tags in the four different protein fragments obtained from the 1D-PAGE and following PEAKS (Zhang et al. 2012) and SPIDER search (Han et al. 2005) indicated a variety of possible proteins. Each of the identified 20 peptides (Table 2) was further analysed.
In a first approach the peptides were searched against the non-redundant protein sequence (nr) entries without taxa limitations. Within these results, no conclusive assignment of the sequences of the bands with the molecular masses 60.5, 63.5, 68.4 or 85.2 kDa to a specific protein and/or to a related barnacle species was possible. In a second approach the search was limited to

| Element | Wt % | Error |
|---------|------|-------|
| C       | 61.11| 0.45  |
| O       | 20.29| 1.06  |
| N       | 16.90| 2.19  |
| S       | 0.56 | 3.36  |
| Mg      | 0.35 | 6.02  |
| Ca      | 0.19 | 10.02 |
| P       | 0.14 | 13.77 |
| K       | 0.10 | 30.86 |

Figure 2. SEM images and EDX spectra of the cement of *D. fascicularis*. (a) EDX spectrum of the elemental composition of the cement float (left). The mean values in weight per cent (Wt %) and the internal error (Error) are shown on the right side. (b) SEM image with the position of the line scan (left). The associated EDX line scan spectrum (right) shows high counts of carbon (C), oxygen (O) and nitrogen (N). Other elements such as sulphur (S) are not detected with this method. (c) SEM image (left) and corresponding dot mappings of the elements C, O, N and S (right). Only C, O and N give fair signals and an even distribution of the elements; S gives a random distribution of noise signals. Scale bar: b, c: 50 μm.
Table 1. Comparison of the amino acid composition (values in residues per hundred) of the cement of *D. fascicularis* with previous data for *D. fascicularis* (Barnes & Blackstock 1974), *Lepas anatifera* (Walker & Youngson 1975), *Megabalanus rosa* (Kamino et al. 1996), *Balanus hameri* and *B. crenatus* (Walker 1972).

| Amino acid | Goose barnacles | Acorn barnacles |
|------------|----------------|-----------------|
|            | *D. fascicularis* | *D. fascicularis* | *L. anatifera* | *M. rosa* | *B. hameri* | *B. crenatus* |
| AsX        | 9.30            | 10.12           | 8.75           | 9.066     | 7.81       | 8.28         |
| Thr        | 4.80            | 6.35            | 5.25           | 7.049     | 6.56       | 6.23         |
| Ser        | 6.58            | 9.88            | 8.75           | 9.905     | 11.36      | 7.69         |
| GlX        | 10.30           | 11.41           | 9.10           | 9.149     | 9.05       | 8.63         |
| Gly        | 4.21            | 8.71            | 9.57           | 7.920     | 8.27       | 8.59         |
| Ala        | 5.60            | 9.88            | 8.99           | 7.473     | 6.87       | 6.47         |
| Val        | 6.05            | 7.76            | 6.65           | 7.251     | 2.74       | 2.19         |
| Met        | 0.49            | 0.59            | 0.56           | 1.600     | 0.72       | 0.67         |
| Ile        | 5.72            | 6.82            | 5.91           | 5.301     | 4.43       | 5.34         |
| Leu        | 8.13            | 9.76            | 9.79           | 8.276     | 8.78       | 8.11         |
| Tyr        | 2.65            | 0.12            | 3.69           | 4.184     | 4.92       | 5.38         |
| Phe        | 5.06            | 4.94            | 3.69           | 3.709     | 3.67       | 3.98         |
| His        | 0.43            | 0.47            | 2.70           | 1.329     | 2.28       | 2.16         |
| Lys        | 2.25            | 2.47            | 3.85           | 5.666     | 5.47       | 6.79         |
| Arg        | 6.42            | 6.12            | 6.07           | 5.602     | 5.85       | 6.13         |
| Pro        | 4.32            | 4.35            | 4.99           | 4.916     | 8.39       | 6.06         |
| Cys/2      | 0.67            | 0.24            | 1.10           | 1.603     | 6.81       | 7.28         |
| Trp        | 0.05            | –               | 0.56           | –         | –          | –            |
| Protein content | 84%            | 75.9%           | 96%            | >90%      | 85.9%      | 84.5%        |

Figure 3. 1D-PAGE and 2D-PAGE of the solubilised *D. fascicularis* cement proteins. (a) 1D-PAGE. Excised gel bands at 85.2 kDa (Dfcp-85), 68.4 kDa (Dfcp-68), 63.5 kDa (Dfcp-63), 60.5 kDa (Dfcp-60) of the coomassie-stained gel after 1D-PAGE of the final soluble fraction of the cement of *D. fascicularis*. 10 μg and 20 μg of protein were applied to the 1D gel. (b) 2D-PAGE. Corresponding positions of the excised protein bands in the coomassie-stained gel after 2D-PAGE of the final soluble fraction after TCA acetone precipitation of the cement.
Table 2. *De novo* peptide sequences and PEAKS® results gained from sequence analysis of protein bands obtained from PAGE of the cement of *D. fascicularis* by MS/MS analysis.

| Dfcp  | Scan | Sequence                  | TLC (%) | ALC (%) | m/z      | z  | ppm       | Local confidence (%) |
|-------|------|---------------------------|---------|---------|----------|----|-----------|-----------------------|
| Dfcp-85 | 1,212 | HPLLSSLNLVSR              | 10.8    | 83      | 696.8984 | 2  | −4.6      | 78 77 88 96 96 91 86 72 87 89 94 95 92 95 93 93 96 |
| Dfcp-85 | 994   | FNVAFDELAATR              | 10.2    | 85      | 677.3482 | 2  | 7.0       | 62 56 80 82 88 89 92 95 95 93 93 96 |
| Dfcp-85 | 1,279 | RRGLVLISLAQPK             | 10.6    | 82      | 737.9545 | 2  | 3.6       | 80 83 80 78 86 92 93 92 84 84 70 87 51 |
| Dfcp-85 | 1,330 | SSVPSELELSDAPEK           | 12.8    | 75      | 894.4531 | 2  | −4.5      | 87 90 90 49 42 61 79 90 83 86 93 94 89 84 70 63 21 |
| Dfcp-85 | 822   | TIPPAAMEELATDK            | 10.4    | 80      | 687.3303 | 2  | −5.9      | 21 86 86 92 90 84 85 89 88 90 84 85 54 |
| Dfcp-68 | 1,847 | FASADELDDLDTVK            | 12.2    | 81      | 826.4038 | 2  | −3.5      | 45 49 70 88 91 95 96 94 94 92 91 81 83 49 |
| Dfcp-68 | 2,911 | RLAAVLSR                  | 7.8     | 86      | 824.8160 | 2  | −6.6      | 79 84 91 93 93 86 88 66 |
| Dfcp-68 | 3,169 | LGSGYVDFLR                | 8.1     | 81      | 563.8023 | 2  | 7.4       | 78 78 89 89 89 90 92 90 90 24 |
| Dfcp-68 | 1,797 | LSQTQILPRA                | 6.6     | 82      | 457.2794 | 2  | 2.0       | 86 87 93 92 80 93 96 24 |
| Dfcp-68 | 2,245 | RSQEVTSWSSKVR             | 11.0    | 78      | 775.4098 | 2  | 2.8       | 76 78 87 88 94 93 84 84 85 88 87 56 57 30 |
| Dfcp-63 | 4,320 | WVTSAWSKAR                | 9.0     | 82      | 639.8329 | 2  | −0.3      | 78 76 89 90 89 91 93 92 75 76 41 |
| Dfcp-63 | 5,370 | FEDFLVNLNFAFSR            | 11.4    | 81      | 843.4136 | 2  | −4.8      | 54 56 77 91 96 97 88 80 86 84 93 87 87 54 |
| Dfcp-63 | 5,066 | ELYGGLTDELTK              | 10.6    | 76      | 761.9024 | 2  | −1.4      | 25 81 68 90 87 91 91 92 88 83 74 72 25 |
| Dfcp-63 | 4,765 | RLEQLAGGKR                | 8.5     | 85      | 564.3314 | 2  | −7.7      | 90 89 90 65 88 89 85 82 83 86 |
| Dfcp-63 | 4,626 | GPDEYELQPR                | 8.1     | 81      | 603.2953 | 2  | 3.1       | 73 84 84 91 74 91 92 80 80 59 |
| Dfcp-60 | 6,857 | SFLADVLGR                 | 7.4     | 82      | 489.2779 | 2  | 7.4       | 48 53 91 94 92 93 94 94 73 |
| Dfcp-60 | 6,795 | KEAALEDFAVSR              | 9.8     | 82      | 677.3605 | 2  | −1.6      | 69 69 91 93 92 93 91 90 87 86 90 26 |
| Dfcp-60 | 6,023 | MMVSNSKVR                 | 7.4     | 82      | 526.2734 | 2  | 0.8       | 75 82 82 86 85 75 85 90 70 |
| Dfcp-60 | 6,453 | FGALGLSRR                 | 6.7     | 84      | 410.7344 | 2  | −7.4      | 91 90 92 85 83 85 88 57 |
| Dfcp-60 | 6,697 | TPLSLESVTR                | 8.4     | 84      | 551.8099 | 2  | 2.0       | 90 92 91 87 87 91 88 80 77 49 |

Dfcp: *D. fascicularis* cement protein (followed by the molecular mass). 'Local confidence is the confidence that a particular amino acid is present in the *de novo* peptide at a particular position. It is presented as a percentage. Total local confidence (TLC) is the sum of the local confidence scores (0 to 1) from each amino acid in the peptide sequence. Average local confidence (ALC) is the average of the TLC. It is TLC divided by the number of amino acids in the peptide sequence. ppm = precursor mass error, calculated as $10^6 \times (\text{precursor mass} - \text{peptide mass}) / \text{peptide mass}$. m/z = precursor mass-to-charge ratio. z = precursor charge.' Source: PEAKS® user manual (http://www.bioinfor.com/peaks/support/).
the taxon barnacles. Table S1 shows the summarised results of over 2,000 hits. The first two identified proteins of each Dfcp peptide with the lowest E-value were displayed. Other results with higher E-values (= less reliable) are exemplarily shown to display possible homologue sequences in other barnacle proteins. It must be remembered that, due to the short peptide sequences, false positive results can occur.

Within the identified proteins the barnacle cement proteins cp-100, cp-20 and cp-19 were found, but never with the lowest E-value. In addition, the cement proteins were spread over the Dfcp6 like following: Dfcp-60 with 1x cp100; Dfcp-63 with 7x cp100 and 1x cp19; Dfcp-68 with 2x cp100, 1x cp20 and 1x cp19; Dfcp-85 with 4x cp100 and 1x cp19 matches. E-values <1 were found only for the Dfcp60 (TPLSLESVTR) matching ‘neurofibrin’, Dfcp-63 (WVTSAWSSKAR) matching ‘lectin BRA-2’, Dfcp-68 (FASADEDDLLDTVK) matching ‘clathrin heavy chain’ and Dfcp-85 (RGRGLVLSHLAQPK) matching ‘settlement inducing protein complex’ (Table S1). Furthermore, >20 times sequences from the barnacle protein ‘MULTIFUNCin’ were identified.

**FTIR spectroscopy**

The analysis showed significant peaks corresponding to proteins containing a β-sheet structure (amide I between 1,640 and 1,620 cm\(^{-1}\)) and/or amyloid-like structure (amide I between 1,630 and 1,610 cm\(^{-1}\)) (Barlow & Wahl 2012). Distinct peaks at 1,517 cm\(^{-1}\) and 1,446 cm\(^{-1}\) in the spectrum of the dried and D\(_2\)O equilibrated sample respectively indicated amide II vibration in agreement with the results of Omoike & Chorover (2004) and Barlow and Wahl (2012). The proteinaceous cement that was analysed did not contain detectable amounts of phosphate (1,253 cm\(^{-1}\)) (Gremlich & Yan 2001). Multiple peaks between 1,236 cm\(^{-1}\) and 950 cm\(^{-1}\) corresponded to amide III vibration (1,234 cm\(^{-1}\)) (Cai & Singh 2004). In addition, vibrations of polysaccharides (1,150–1,000 cm\(^{-1}\)) and phosphodiester bonds (1,230–950 cm\(^{-1}\)) (Omoike & Chorover 2004) were also found. Due to the lack of detectable bands in the region between 1,000 and 700 cm\(^{-1}\) characteristic for phosphate-sugar backbone vibrations (Socrates 2001), the peaks at 1,076 and 1,055 cm\(^{-1}\) were assigned to polysaccharide vibrations rather than phosphodiester bonds (Figure 4a, Figure S3).

**Raman spectroscopy**

The results confirmed the proteinaceous nature of the cement containing a significantly higher content of β-sheet structure than β-turn, α-helix and random coil (1,680–1,665 cm\(^{-1}\)) (Jiskoot & Crommelin 2005). The proteins contained a significant amount of phenylalanine (1,003 cm\(^{-1}\) and 1,606 cm\(^{-1}\)) (Jiskoot & Crommelin 2005; Severcan & Haris 2012) and mostly buried tyrosine residues (855 and 832 cm\(^{-1}\)) (Severcan & Haris 2012). Bands for L-DOPA (735–730 cm\(^{-1}\)) (Oka & Garrell 2000) and disulphide bridges (550–500 cm\(^{-1}\)) (Severcan & Haris 2012) were not found by Raman spectroscopy indicating an absence or an amount below the sensitivity of the method (Figure 4b). L-DOPA was also not detected on staining the cement with the Arnow method (Figure S2b).

**Discussion**

*D. fascicularis* is the only barnacle which produces gas-filled cement, thereby allowing the animal to float. The animals occur either individually or in clusters attached to flotsam or to the cement-buoys of conspecifics. The cement consisted of 8% dry matter and had a high water content of 92% in total, which fulfilled the definition of a hydrogel (Zavan et al. 2009). Other adhesives have also been classified as hydrogels, for example the gastropod adhesive gels (Smith 2006), the adhesive skin exudates of the Australian frog *Notaden bennetti* (Graham et al. 2005), the prey capture glue of the velvet worm *Euperiopatoides* sp. (Graham et al. 2013) and the egg attachment glue of the moth *Opodiphthera* sp. (Li et al. 2008), but none of these have a foam-like structure as found in *Dosima*.

In agreement with the organic nature of the cement and the results of the adhesives of other barnacle species (Berglin & Gatenholm 2003; Sullan et al. 2009) the EDX spectra of the cement float of *D. fascicularis* showed high counts of carbon (C), oxygen (O) and nitrogen (N). However, in *D. fascicularis* the elements were distributed evenly, unlike the cement of *Amphibalanus amphitrite* where high amounts of C, N and O were concentrated in rod-shaped structures (Sullan et al. 2009). Sulphur (S), magnesium (Mg) and small amounts of calcium (Ca), phosphorus (P) and potassium (K), presumably originating from seawater, were detected in the EDX spectra of the cement, but not in the line scans and dot mappings. This indicated that the quantities of these elements were near the detection limit of the EDX-system, which was 0.1 wt %. Interestingly, NaCl was not seen in the EDX spectra. The reason could be that the cement was rinsed in fresh water before it was used for EDX investigations and NaCl was washed away.

High amounts of Ca have been found in the cement of balanoid barnacles with a calcareous base (Walker 1972; Sangeetha et al. 2010). It is possible that Ca in the cement of these species came from the basal plate and/or the edge of the calcareous shell (Sangeetha & Kumar 2011). This kind of distortion could be ruled out in *D. fascicularis* because this goose barnacle has a membranous attachment disc and its calcareous plates do not come into contact with the cement.

Although sulphur was identified in the EDX spectra of the cement of *D. fascicularis* no disulphide bridges
Figure 4. FTIR and Raman spectra of dry and wet *D. fascicularis* cement. (a) FTIR spectrum of the cement. The region between 4,000 and 500 cm$^{-1}$ is magnified. Blue: sample equilibrated in D$_2$O; Red: dry sample. (b) Raman spectrum of the cement. The region between 4,000 and 480 cm$^{-1}$ is magnified. Blue: sample equilibrated in D$_2$O; Red: dry sample.
could be detected with Raman spectroscopy. In acorn barnacle cement, disulphide bonds are known to stabilise the protein complex and they contribute to its insolubility (Naldrett 1993; Kamino et al. 2000). Disulphide bonds are also commonly found in the adhesives of echinoderms, mussels, snails and sabellariid polychaetes (Benedict & Waite 1986; Flammang et al. 1998; Smith et al. 1999; DeMoor et al. 2003; Zhao et al. 2005).

Sulphur can also occur as sulphated polysaccharides as seen in the adhesive of the sandcastle worm Phragmatopoma californica (Wang & Stewart 2013). A small distinct peak at 1,055 cm$^{-1}$ in the FTIR spectrum of the $D. fascicularis$ cement equilibrated in D$_2$O corresponds to polysaccharide vibration, which suggests that some glycosylated proteins occur in the cement. This was confirmed by the PAS method and the total carbohydrate analysis.

With the EDX system, phosphorus was detected in small amounts in the cement of $D. fascicularis$, which has only been reported for other barnacle cement by Walker (1972). Phosphorus is required as an activation component for post-translational modification to form phosphoserine, which plays an important role in the formation of a strong adhesive. This component was also found in the adhesives of caddis flies, mytilid mussels, sandcastle worms and the Cuvierian tubules of the sea cucumber as well as in kelp spore adhesive (Waite & Qiu 2001; Zhao et al. 2005; Flammang et al. 2009; Stewart & Wang 2010; Petrone et al. 2011). However, phosphoserines were not found in the adhesive system of barnacles (Kamino 2010) and could not be chemically verified in $D. fascicularis$. Also L-DOPA, a post-translational modification of tyrosine, has never been identified in barnacle cement (Naldrett 1993; Kamino et al. 1996). This result was confirmed by the Arnow staining and the FTIR and the Raman spectra of the cement of $D. fascicularis$.

The amino acid composition of the cement of $D. fascicularis$ differed from earlier data by Barnes and Blackstock (1974, 1976), mainly in the smaller quantities of alanine, glycine and serine and the larger quantity of tyrosine. Tyrosine ring vibration is also indicated at 1,517 cm$^{-1}$ in the FTIR spectra (Barth & Zscherp 2002) and as doublets at 833 and 855 cm$^{-1}$ in the Raman spectra (Severcan & Haris 2012). Comparing the Dosima cement with that of $L. anatifera$, $M. rosa$, $B. hameri$ and $B. crenatus$ (Walker 1972; Walker & Youngson 1975; Kamino et al. 1996), the main differences are the smaller amounts of alanine, glycine, histidine, lysine and serine. The values of phenylalanine in the Dosima cement were higher, which was confirmed by Raman spectroscopy. Comparing the Dosima cement with that of $B. hameri$ and $B. crenatus$ (Walker 1972), the amount of valine was considerably higher and that of cystine and proline smaller (see Table 1). The high amounts of hydrophobic amino acids found in the cement of $D. fascicularis$ agree with the findings in the cement of $B. crenatus$, $B. perforatus$ and $M. rosa$. Naldrett & Kaplan (1997), Kamino (2010) and Kamino et al. (2012) stated that hydrophobic interactions rendered the cement matrix insoluble and made it resistant to decomposition by

The cement of the closely related goose barnacle $Lepas anatifera$ (Walker & Youngson 1975) and that of the acorn barnacle $Megabalanus rosa$ (Kamino et al. 1996) had a higher protein content (>90%) than $D. fascicularis$.

According to Barlow et al. (2010), barnacle cement is largely composed of fibrillar proteinaceous material. Fibrous structures were identified by microscopic analyses in the cement of acorn barnacles (Wiegemann & Watermann 2003; Dickinson et al. 2009; Sullan et al. 2009) and the goose barnacle $D. fascicularis$ (Zheden et al. 2012). One fibrillar proteinaceous structure associated with bioadhesion in barnacles is amyloid (Kamino 2008; Sullan et al. 2009; Barlow et al. 2010). In $D. fascicularis$, amyloid was detected by histochemical methods in the cement glands and in the cement (McEvilly 2011) but it was not found in the closely related barnacle $Lepas anatifera$ (Jonker et al. 2012).

The FTIR spectrum of $D. fascicularis$ cement indicated the probability of a mixture of cross, parallel and antiparallel β-sheets due to major overlap of the relevant areas for amyloid-like and β-sheet structures (1,610–1,630 cm$^{-1}$ vs 1,620–1,640 cm$^{-1}$), which could not be further specified by processing the amide I region via deconvolution (Barlow & Wahl 2012) (not shown). The β-sheet structures were previously found to be a feature of barnacle adhesives (Sullan et al. 2009; Burden et al. 2012). Furthermore, the FTIR spectra of wet and dry cement differed in the amide I band shift (Figure S3), which was most likely due to refolding of the secondary structure caused by dehydration (Hédoux et al. 2012; Hartwig et al. 2013).

The amino acid composition of the cement of $D. fascicularis$ differed from earlier data by Barnes and Blackstock (1974, 1976), mainly in the smaller quantities of alanine, glycine and serine and the larger quantity of tyrosine. Tyrosine ring vibration is also indicated at 1,517 cm$^{-1}$ in the FTIR spectra (Barth & Zscherp 2002) and as doublets at 833 and 855 cm$^{-1}$ in the Raman spectra (Severcan & Haris 2012). Comparing the Dosima cement with that of $L. anatifera$, $M. rosa$, $B. hameri$ and $B. crenatus$ (Walker 1972; Walker & Youngson 1975; Kamino et al. 1996), the main differences are the smaller amounts of alanine, glycine, histidine, lysine and serine. The values of phenylalanine in the Dosima cement were higher, which was confirmed by Raman spectroscopy. Comparing the Dosima cement with that of $B. hameri$ and $B. crenatus$ (Walker 1972), the amount of valine was considerably higher and that of cystine and proline smaller (see Table 1). The high amounts of hydrophobic amino acids found in the cement of $D. fascicularis$ agree with the findings in the cement of $B. crenatus$, $B. perforatus$ and $M. rosa$. Naldrett & Kaplan (1997), Kamino (2010) and Kamino et al. (2012) stated that hydrophobic interactions rendered the cement matrix insoluble and made it resistant to decomposition by
marine bacteria (Naldrett 1993). Both qualities are particularly important for Dosima cement because the cement needs stability against mechanical impact while drifting in the sea (Zheden et al. unpublished). Because, unlike other barnacles, an excess amount of cement is produced by the animal, most of the cement surface is exposed to the environment and thus also to bacteria. Kamino (2008) reported that the major bulk proteins of barnacle cement are hydrophobic, whereas proteins for surface functions are hydrophilic. Barlow et al. (2009) indicated that the cement of live barnacles was moderately hydrated and therefore hydrophilic in its natural state. The isoelectric point of the observed cement proteins of D. fascicularis ranged from pH 3.5 to 6.0, indicating that mainly acidic proteins could be solubilised.

Indistinct protein bands with a molecular weight from <10.0 to 90.0 kDa were found in the cement of D. fascicularis by Barnes & Blackstock (1976). In the present study protein bands with a molecular weight between 47.4 and 250.0 kDa were identified. N-terminal sequence analysis by EDMAN degradation failed due to blocked termini of the proteins (data not shown). The reason for the N-terminal blocking can be by natural processes as protection for example against bacterial proteolytic enzymes or because of an undesirable effect of the sample preparation (eg by impurities of solubilisation or PAGE chemicals) (Wellner et al. 1990).

De novo sequencing was originally performed mainly manually with the help of database search, but is now commonly carried out by computational systems using algorithms to reduce time and costs (Ma & Johnson 2012). In the present study a set of 20 peptide sequences was identified (five of each analysed Dcfp). A NCBI blastp search of each of the peptides showed no results that allowed definite conclusions to be drawn about the analysed Dcfp or the underlying mechanisms of the adhesion process. In addition, it must be taken into account that only some of the solubilised proteins were analysed. Some of the identified peptides showed similarities to the barnacle cement proteins (cp) (Kamino 2013). At least 14 sequence hits were found for cp100 k, three hits for cp19 k and two for cp20 k and no hit for cp68 k or cp52 k. Markedly, the most abundant cement protein cp100 k (Kamino 2013; Lin et al. 2014) was also found in the present study with most matches (but not with the lowest E-values).

The NCBI blastp showed more than 20 matches for the protein MULTIFUNCin. This ~1,500 amino acid large glycoprotein (accession no: AFY13480) functions within the biomineralisation of barnacle shell and as attractant for the larvae of the barnacle. The latter applies also to Dosima whose larvae settle on the cement of con-specifics. However, MULTIFUNCin was not mentioned in the context of cement formation (Ferrier 2010).

The poor coverage of the database searches used could indicate unexpected post-translational modifications or peptide mutations (Ma & Johnson 2012), but it could also imply that in addition to the known cement proteins some unknown proteins are part of the cement. The applied MS/MS techniques can be used as the basis for understanding the Dosima cement as a natural underwater adhesive.

Understanding more about the Dosima cement could lead to the production of non-toxic artificial glues for medical and technical purposes (Smith & Callow 2006; von Byern & Grunwald 2010). In a follow-up study, it is planned to use peptide sequence tags to identify the full-length proteins by genome or transcriptome sequencing, or by RT-PCR methods (Hennebert et al. 2012; Lin et al. 2014). Furthermore, the peptides characterised in this study will be used to identify possible inorganic surface binding sequences in a combined computational and experimental approach as described by Steckbeck et al. (2014). The peptides can also be the basis for designing fluorescent-labelled nucleic acids for in situ hybridisation probes to localise RNA sequences (Wang & Stewart 2012). In addition, anti-peptide antibodies will be raised to localise the protein using immuno histochemistry approaches.

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