Isolation and biochemical characterization of underwater adhesives from diatoms

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Many aquatic organisms are able to colonize surfaces through the secretion of underwater adhesives. Diatoms are unicellular algae that have the capability to colonize any natural and man-made submerged surfaces. There is great technological interest in both mimicking and preventing diatom adhesion, yet the biomolecules responsible have so far remained unidentified. A new method for the isolation of diatom adhesive material is described and its amino acid and carbohydrate composition determined. The adhesive materials from two model diatoms show differences in their amino acid and carbohydrate compositions, but also share characteristic features including a high content of uronic acids, the predominance of hydrophilic amino acid residues, and the presence of 3,4-dihydroxyproline, an extremely rare amino acid. Proteins containing dihydroxyphenylalanine, which mediate underwater adhesion of mussels, are absent. The data on the composition of diatom adhesives are consistent with an adhesion mechanism based on complex coacervation of polyelectrolyte-like biomolecules.

Keywords: Amphora coffeaeformis; Craspedostauros australis; diatom; adhesive material; 3,4-dihydroxyproline; uronic acids

Introduction

A diverse range of animals and plants (eg mussels, barnacles, carnivorous plants, insects and algae) have the ability to adhere to natural or man-made surfaces through the secretion of adhesive substances (bioadhesives) (see Smith & Callow 2006). The performance of such bioadhesives (eg for self-healing or adhesion under water) is often superior to synthetic glues (Smith & Callow 2006). However, due to the lack of knowledge on the chemical structures of most bioadhesives, the molecular mechanisms underlying their adhesive properties have remained largely enigmatic. To date, the adhesive pads of the marine mussel byssus are by far the best studied bioadhesive system, which depends on proteins containing numerous dihydroxyphenylalanine (Dopa) residues. Dopa serves both for intermolecular covalent cross-linking and metal coordination cross-linking, as well as interaction with the surface (Hwang et al. 2010; Lee et al. 2011). Although the exact molecular mechanism is still under debate, ongoing investigations have started to reveal insights into the structure–function correlation in Dopa-containing adhesive proteins and the importance of the redox potential of the immediate environment in controlling their adhesive properties (Yu et al. 2011; Danner et al. 2012; Hwang et al. 2012; Nicklisch & Waite 2012; Lu et al. 2013).

An impressive example of biological underwater adhesion is provided by diatoms, which are able to reversibly adhere and colonize almost any type of natural or man-made surface. Diatoms are a large group of eukaryotic microalgae that have a rigid cell wall made of silica (SiO₂). Diatoms are often among the first organisms to colonize underwater surfaces, because their adhesive mucilage enables the cells to attach to substrata of a very wide range of chemical composition (organic, inorganic, natural or man-made) (for reviews see Molino & Wetherbee 2008; Molino et al. 2009). The colonization of man-made structures, termed biofouling, causes a substantial problem due to increased drag on water-borne vessels and thus increased fuel consumption (Callow & Callow 2011; Fitridge et al. 2012). In 2008, the International Maritime Organization banned the use of toxic tributyltin oxide containing antifouling (AF) paints, which has spurred the development of a new generation of AF technologies that employ either biocidal coatings (both copper containing and copper-free) or fouling-release (FR) coatings. Biocidal coatings aim to prevent initial attachment of organisms, while FR coatings are intended to reduce the strength of biofilm attachment allowing for ‘self-cleaning’ due to hydrodynamic shear forces generated by the ship’s movement (for review see Callow & Callow 2011). Although some FR coatings are effective in the prevention of animal and macrofouling, they have so far been unsuccessful in preventing the accumulation of diatom-containing biofilms (Molino et al. 2009; Dobretsov & Thomason 2011; Zargiel et al. 2011; Zargiel & Swain 2014). Given the tenacity of diatom
adhesion to non-toxic FR coatings, understanding the molecular mechanism of this process should be instrumental in the development of coatings that can prevent the attachment of diatoms.

Surface adhesion of diatoms belonging to the raphid pennate type requires the secretion of so-called adhesive mucilage strands through a dedicated slit in the silica cell wall, which is termed the raphe (Drum & Hopkins 1966). Adhering cells can then move across the surface, which is accomplished through an actin-myosin dependent cytoskeletal motor that translocates the adhesive mucilage strands in a rearward direction through the raphe, thereby moving the cell forward (Edgar & Pickett-Heaps 1983; Edgar & Zavortink 1983; Edgar & Pickett-Heaps 1984; Poulsen et al. 1999). When the adhesive mucilage strands reach the end of the raphe they are released and left as a trail behind the moving cell (for reviews see Edgar & Pickett-Heaps 1984; Hoagland et al. 1993; Wetherbee et al. 1998).

To date only sparse information is available on the molecular composition of the adhesive mucilage produced by diatoms. Histochemical and lectin staining indicate the presence of negatively charged polysaccharides (Daniel et al. 1980; Wigglesworth-Cooksey & Cooksey 2005), and AFM studies suggest that the adhesive contains (glyco)proteins with modular domains (Dugdale et al. 2005, 2006). A monoclonal antibody, StF.H4, raised against cell wall-associated components from the diatom *Craspedostauros australis*, bound to both the cell wall surface and the trails of the adhesive mucilage, and also inhibited cell adhesion (Lind et al. 1997). The epitope recognized by StF.H4 involves a carbohydrate moiety, but its chemical structure has so far remained unidentified (Chiovitti et al. 2003). The main obstacle for characterizing the chemical structure of the diatom adhesive mucilage has been the lack of a method for its isolation, free of contaminating cellular material. A number of previous studies have attempted to characterize diatom extracellular polymeric substances by performing gentle extractions of whole cells (Staats et al. 1999; de Brouwer & Stal 2001, 2002; Khandeparker & Bhosle 2001; Khodse & Bhosle 2010). However, as Chiovitti et al. (2004) demonstrated, these preparations are typically dominated by glucose due to contamination from intracellular stores of chrysolaminarin. More recently, characterization of the carbohydrate composition of the adhesive mucilage from the diatom *Phaeodactylum trihornum* was attempted. Cells adhering to coverslips were scraped off with a razor blade, yet fluorescence microscopy demonstrated that numerous damaged and intact cells remained on the scraped coverslips as indicated by chloroplast autofluorescence (Willis et al. 2013). Carbohydrate analysis of the remaining material on the coverslips detected mannose (33 mol%), glucose (19 mol%), galactose (17 mol%), xylose (12 mol%), rhamnose (10 mol%), and arabinose (6 mol%). However, it is currently unknown what fraction of these carbohydrates was contributed by the cellular contaminants rather than the adhesive mucilage.

In this paper, a new method for the isolation of adhesive mucilage from the raphid pennate diatoms *C. australis* and *Amphora coffeaeformis* is presented. Both *C. australis* (Higgins et al. 2003; Holland et al. 2004; Molino et al. 2006; Willis et al. 2007) and *A. coffeaeformis* (Wustman et al. 1997; Finlay et al. 2002; Holland et al. 2004; Molino et al. 2006; Hodson et al. 2012) have served as model diatoms in previous biofouling research. It is demonstrated that the isolated adhesive mucilage is essentially free of cellular contaminants, thus allowing for the determination of its carbohydrate and amino acid composition with unprecedented accuracy.

**Materials and methods**

**Culture conditions**

*Craspedostauros australis* Cox clone CCMP3328 and *Amphora coffeaeformis* (Agardh) Kuetzing clone CCMP126 were grown in an artificial seawater medium according to the North East Pacific Culture Collection (http://www3.botany.ubc.ca/cccm/NEPCC/esaw.html), intermittently supplemented with 100 μg ml⁻¹ of penicillin and 100 μg ml⁻¹ of streptomycin, at 18°C under constant light an intensity between 40 and 60 μmol photons m⁻² s⁻¹, using cool white and warm white fluorescent tubes as the light source.

**Chemicals**

Monosaccharide (glucose, galactose, mannose, xylose, fucose, fructose, arabinose, rhamnose, ribose, galactosamine, glucosamine, glucuronic acid, galacturonic acid and gluconic acid) and amino acid standards were purchased from Sigma and for AAA-direct amino acid analyses from Thermo Scientific Dionex (Idstein, Germany). Trihydroxyproline (Dihyp) were kindly provided by Carol Taylor (Louisiana State University). 2,3-Dihydroxyproline (cis-Dihyp) and 2,3-trans-3,4-trans-dihydroxyproline (trans-Dihyp) were prepared from a commercial 50% w/w stock (Fisher, Schwerte, Germany). Sodium acetate for monosaccharide analyses was purchased from Sigma-Aldrich Chemie (Munich, Germany). Sodium hydroxide (NaOH) solutions for high performance anion exchange chromatography (HPAEC) were prepared from a commercial 50% w/w stock (Fisher, Schwerte, Germany). Sodium acetate for monosaccharide analyses was purchased from Sigma and for AAA-direct amino acid analyses from Thermo Scientific Dionex (Idstein, Germany). Trifluoroacetic acid (TFA), hydrochloric acid (HCl) and phenylisothiocyanate (PITC) were purchased from Thermo Scientific. NaH¹³CO₃ was purchased from Cambridge Isotope Laboratories (MA, USA). 2,3-trans-3,4-cis-dihydroxyproline (cis-Dihyp) and 2,3-cis-3,4-trans-dihydroxyproline (trans-Dihyp) were kindly provided by Carol Taylor (Louisiana State
University, LA, USA). Ultrapure H2O (MilliQ) was used for all experiments.

**Staining of diatom adhesive trails**

To visualize the adhesive trails, the growth medium was removed and substrata were overlaid with a solution containing 1 mg ml⁻¹ of Stains-All (Sigma-Aldrich) in formamide. Following 15 min incubation in the dark, the Stains-All solution was removed and the substratum was washed three times with MilliQ H2O. The trails were then lyophilized to dryness and stored at −20°C. The isolated adhesive material was hydrolyzed with 6 M HCl containing 10% phenol for 24 h at 110°C. The hydrolysate was then evaporated to dryness in a vacuum centrifuge at 50°C. The amino acids were either directly analyzed on an Aminopac PA-10 column (Thermo Scientific Dionex) using HPAEC-PAD, or they were reacted with PITC and the resulting phenylthiocarbamoyl (PTC) derivatives separated on a 250/4 Nucleosil 100-3 C18 column (Machery Nagel, Düren, Germany) using reverse phase high performance liquid chromatography (HPLC) with UV detection (254 nm). The eluents for HPAEC-PAD were 10 mM NaOH (eluont A), 250 mM NaOH (eluont B), and 1 M sodium acetate, 25 mM NaOH (eluont C). The gradient programs for separation of amino acids by HPAEC-PAD are shown in Supplementary Tables S2 and S3, respectively. The eluents for RP-HPLC were 50 mM sodium phosphate buffer, pH 6.4 in 50% acetonitrile (eluont B). The gradient program used for RP-HPLC is shown in Supplementary Table S4. Cysteine and methionine were detected as cysteic acid and methionine sulfone after the isolated adhesive material was oxidized with performic acid prior to acid hydrolysis. The tryptophan content was determined following hydrolysis of the adhesive material in 2 M NaOH for 2 h at 110°C and subsequent analysis by HPAEC-PAD. To investigate the Dopa content of the adhesive material, hydrolyzed and evaporated samples were resuspended in sample dilution buffer and run on a post-column ninhydrin-based amino acid analyzer (Dopa detection limit is 50 pmol) (Sykam S433, Fürstenfeldbruck, Germany).

**FTMS analysis of PTC-derivatized amino acids**

The PTC derivatives of \( \text{trans} \)-Dihyp and \( \text{cis} \)-Dihyp were synthesized by reaction with PITC, and \( \text{PTC-trans} \)-Dihyp, and \( \text{PTC-cis} \)-Dihyp were collected as fractions from RP-HPLC runs using a phosphate-buffered acetonitrile gradient as described above under ‘Amino acid analysis’. Each fraction was then desalted by RP-HPLC using an Everest 238EV52 C18 column (Grace Silica GmbH, Düren, Germany), and a TFA/H2O/
acetonitrile gradient (0–70% B in 30 min, flow rate: 0.2 ml min⁻¹; eluent A: 0.1% TFA in H₂O, eluent B: 0.08% TFA in acetonitrile). Fractions containing PTC-derivatives were collected, lyophilized to dryness, and the residues stored at −80°C. For FTMS analysis the residues were dissolved in 50% MeOH, 0.1% formic acid, and directly infused into a SolarixX 12 T FTMS instrument using an Apollo II Dual ESI/MAL ion source (Bruker Daltonik GmbH, Bremen, Germany). Measurements were performed in the positive ion mode, tuned for mass range m/z 100–600, and calibrated externally with arginine clusters.

**Quantifying cellular contamination of the adhesive material**

The silica content of whole cells and the isolated adhesive material was determined by dissolving the samples in 2 M NaOH at 95°C for 30 min and then performing a colorimetric assay (Baumann 1960). The total mass of individual cells was estimated as follows. Known numbers of cells (8.5 × 10⁶ for *C. australis*, 7.5 × 10⁶ for *A. coffeaeformis*) were pelleted by centrifugation (5 min, 20,000 g). To remove adhering seawater, the cell pellets were briefly (<1 min) resuspended in H₂O and pelleted again (5 min, 20,000 g). The cell final pellets were lyophilized and weighed. Cell numbers were determined using a Biorad TC20 cell counter (Biorad, Munich, Germany) for *A. coffeaeformis* and a Thoma hemocytometer for *C. australis*.

**Results**

**Isolation of diatom adhesive material**

To allow for the deposition of diatom adhesive on surfaces, aggregate-free cell suspensions of *C. australis* and *A. coffeaeformis* were seeded onto polystyrene Petri dishes and cultivated overnight. During this time the cells adhered to the surface as a monolayer, and deposited trails consisting of the adhesive material (Figure 1a, d). Subsequently, removal of the cells from the surface was accomplished by spraying controlled jet pulses of water (delivered by a ‘water flosser’ apparatus) over the entire Petri dish surface. Removal of *A. coffeaeformis* cells required a higher water pressure than removal of *C. australis*, which is consistent with the previously reported higher adhesion strength of *A. coffeaeformis* (Holland et al. 2004). The water spraying removed the cells efficiently from the Petri dish surface, but not the adhesive trails, as was demonstrated by light microscopy after staining the trails with the dye ‘Stains-All’ (Campbell et al. 1983) before (Figure 1a, d) and after (Figure 1b, e) water spraying. Following removal of the cells, the adhesive material was recovered by scraping it off the Petri dish surface using a cell lifter. After scraping, staining with ‘Stains-All’ indicated essentially complete removal of the adhesive material from the surface (Figure 1c, f). The scraped-off adhesive material was lyophilized to dryness yielding 24.6 ± 4.5 μg (from *A. coffeaeformis*) and 34.2 ± 6.1 μg (from *C. australis*) of solid material per 100 cm² of dish surface.

**Quantification of cellular contamination**

Inspection by light microscopy indicated almost complete absence of cells from the isolated adhesive material. However, it was a concern that the small amounts of cellular contamination might significantly contribute to the composition of the isolated adhesive material. Therefore, the amount of cellular contamination was quantified as follows. Due to the silica-based cell walls of diatoms, the Si content in isolated adhesive material can be used as a tracer for the presence of cells. Assuming that silicic acid or silica is not a structural component of diatom adhesives, the silica content of the isolated adhesive material would be a direct measure for the number of contaminating cells. Using a routine silica assay (Baumann 1960), 56.7 nmol silica and 118.4 nmol silica were found in 1 mg of dry isolated adhesive material from *A. coffeaeformis* and *C. australis*, respectively. Considering the Si content per cell, 1 mg of the isolated adhesive material contained 7.3 × 10⁴ cells of *A. coffeaeformis* (0.78 ± 0.09 pmol Si per cell) and 7.2 × 10⁴ cells of *C. australis* (1.64 ± 0.17 pmol per cell). This analysis demonstrates that water spraying removed >99.8% of *A. coffeaeformis* and *C. australis* cells from the surface. Taking into account the average mass per cell, the cellular contamination in the isolated adhesive material amounts to <2.1 weight% for *A. coffeaeformis* (0.29 ± 0.005 pg per cell) and <2.3 weight% for *C. australis* (0.32 ± 0.01 pg per cell). Therefore, the isolated adhesive material is almost 98% pure.

**NMR analysis**

Attempts to completely solubilize the isolated adhesive material using detergents, chaotropic agents, high ionic strength, and reducing agents were unsuccessful. As a first step towards investigating the chemical composition of the intact adhesive, solid state ¹³C magic angle spinning (MAS) NMR was employed (note: the adhesive was isolated from ¹³C-labeled cells). The ¹³C MAS NMR spectra for the adhesive from *A. coffeaeformis* and *C. australis* are very similar (Figure 2). The spectrum of the *C. australis* adhesive contains five signal groups (1, 3, 4, 5, 6) that are also present in the spectrum from the *A. coffeaeformis* material. These signal groups correspond to C=O groups (1), aromatic groups (3); the anomeric C-atom in polysaccharides (4), C-O groups (5), and aliphatic groups (6); signal group 2, which is present only
in the A. coffeaeformis spectrum, corresponds to the ε-C of the arginine side chain (ca 160 ppm) (Wishart et al. 1995). Signal group 4 is specific for polysaccharides (Gröger et al. 2009) while signal groups 2 and 3 suggest the presence of proteins. All other signal groups could be due to the presence of both proteins and polysaccharides. By comparing the intensity of signal group 4 with the total signal in each spectrum (Figure S1) and assuming exclusively hexoses as polysaccharide constituents it was estimated that ≥70% of the organic components of the isolated adhesive material from A. coffeaeformis and C. australis are composed of polysaccharide. The other organic components (<30% of the total material) are probably proteins.

**Carbohydrate analysis**

To characterize the carbohydrate component, the isolated adhesive materials from C. australis and A. coffeaeformis were subjected to acid hydrolysis followed by determination of the monosaccharide compositions using high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) (Figure S2). Four different conditions for acid hydrolysis were employed as the yield of neutral compared to amino
sugars and sugar acids can strongly depend on the concentration and type of acid, as well as incubation time and temperature (Figure S3) (Fan et al. 1994). Table 1 shows the monosaccharide compositions of the isolated adhesive materials, listing for each monosaccharide the maximum relative amount that was detected in these analyses. The monosaccharide composition of the adhesive material is rather complex for both diatom species. They contain hexoses, pentoses, deoxyhexoses, and uronic acids. Three monosaccharides (coined X1, X2, X3) remained unidentified in the *C. australis* adhesive, whereas only one component (X1) could not be assigned to known monosaccharides in the *A. coffeaeformis* adhesive. As the isolated adhesive materials contained small amounts of cellular contamination (see above), whether these contributed significantly to the carbohydrate composition of the adhesive materials was investigated. Taking into account the number of contaminating cells that are present per mg of isolated adhesive mucilage (see above), the cellular contaminants accounted for < 10% of the glucose in the *A. coffeaeformis* adhesive (Figure S2a), and for the entire amount of glucose in *C. australis* (Figure S2b). However, the cellular contamination did not contribute to any of the other monosaccharide components (Figure S2a and b, red traces). This indicated that all monosaccharides except glucose are specific constituents of the polysaccharides of the adhesive material from *A. coffeaeformis* and *C. australis*. Given that the amount of glucose in *C. australis* that would result from cellular contamination equals almost twice the amount observed in the adhesive (185%), it is likely that the level of cellular contamination in the isolated adhesive from this species has been overestimated. The contaminating amounts of glucose likely originated from intracellular pools of the storage polysaccharide, chrysolaminarin (ie a β-1,3- and β-1,6-linked glucan) (Chiovitti et al. 2004).

The adhesive materials from both species have similar complexities in monosaccharide composition but exhibit differences regarding their relative quantities (Table 1). The *A. coffeaeformis* adhesive material contains higher relative amounts of hexoses and deoxyhexoses, whereas the relative content of pentoses and uronic acids is substantially higher in the *C. australis* adhesive material. Both species reveal the presence of high amounts of uronic acids, which may be important for stabilizing the adhesive material by ionic cross-linking via the binding of polyvalent cations. Such a role has been suggested for uronic acid rich polysaccharides that are major constituents of the adhesive biofilms of bacteria. Previous studies on the exopolysaccharides isolated from *A. coffeaeformis* and other *Amphora* spp. have revealed the presence of the same types of monosaccharides (Bhosle et al. 1996; Wustman et al. 1997; Khodse & Bhosle 2010). However, the relative molar ratios in these studies are very different from the present data, probably due to the high levels of chrysolaminarin contamination as discussed above.

### Amino acid analysis

The $^{13}$C MAS NMR spectra suggested that proteins are the second largest organic component of the adhesive material from *A. coffeaeformis* and *C. australis* (see Figure 2). To further investigate this, amino acid analyses of the isolated adhesive materials were performed. After acid hydrolysis, the material was either directly analyzed using HPAED-PAD (Figure S4), or derivatized with phenylisothiocyanate (PITC) and then subjected to high performance liquid chromatography (HPLC) on a C18 reversed phase (RP) column (Figure 3). The amino

| Monosaccharide | C. australis | A. coffeaeformis |
|---------------|-------------|-----------------|
| Hexose        | 9.2         | 17.6            |
| Glucose       | 2.3         | 17.7            |
| Mannose       | 9.8         | 2.6             |
| Deoxyhexose   | 1.8         | 17.2            |
| Fucose        | 8.3         | 6.6             |
| Xylose        | 24.9        | 9.1             |
| Rhamnose      | 10.3        | 3.9             |
| Amino sugar   | n.d.        | 0.5             |
| Galactosamine | 18.0        | 1.2             |
| Galacturonic acid | 15.5     | 23.6            |
| Glucuronic acid | +          | +               |
| X1 (22.4 min) | +           | n.d.            |
| X2 (23.9 min) | +           | n.d.            |
| X3 (26.9 min) | +           | n.d.            |

Note that the presence of glucose is mainly due to cellular contaminants. The three unidentified monosaccharide components, X1, X2 and X3 could not be quantified and thus were not included in the mol% calculation (note: in pulsed amperometric detection (PAD) the signal intensity strongly varies with the type of molecule). + =present in unknown quantity, n.d. = not detected.
acid compositions of the adhesive materials were substantially different between the two species (Table 2). The small amount of cellular contamination did not contribute significantly to the amino acid composition of the isolated adhesive materials (Figure 3, dashed traces). Glycine, serine, and threonine constituted about half of the amino acid residues of the adhesive material from *C. australis*, whereas these amino acids were only present at moderate levels (~20 mol% combined total) in the *A. coffeaeformis* adhesive material, which instead was dominated by an exceptionally high content of arginine (27.4 mol%). Combined results from the carbohydrate and amino acid analysis indicate that the carbohydrate to protein ratio (C:P) for *A. coffeaeformis* is ~4:1 whereas for *C. australis* the ratio is ~2:1.

A non-standard amino acid, coined Xa, was present in the adhesive materials from both species, but was much more abundant in *C. australis* (17 mol% vs 3 mol%). To identify the chemical structure of Xa, the PTC-derivatized component was isolated by RP-HPLC, and subjected to high resolution molecular mass analysis using Fourier transform mass spectrometry (FTMS). This revealed a molecular mass of 283.07478 Da (mean error: ± 0.28 ppm) for the singly positively charged ion (PTC-Xa +H )+. Based on the molecular mass and the simulated isotope pattern, the predicted atomic composition of the (PTC-Xa +H )+ ion is C_{12}H_{15}N_{2}O_{4}S (Figure S5a). The presence of a single S-atom on the molecule indicated that it contained only one PTC (C_{7}H_{6}NS) residue, yielding C_{5}H_{9}O_{4}N as the predicted atomic composition of Xa. Furthermore, the sole N atom in Xa must be part of a primary or secondary amino group, because it can be derivatized with PITC.

To gain further insight into the chemical structure of Xa, the (PTC-Xa +H )+ main isotope ion was isolated in the FTMS instrument and subjected to collision-induced (CID) fragmentation (Figure S5c). The resulting spectrum exhibited two main singly positive charged fragmented ions with predicted atomic compositions of C_{5}H_{10}NO_{4} (148.06045 Da ± 0.1 ppm) and C_{4}H_{8}NO_{2} (102.05498 Da ± 0.25 ppm). The 148.06045 Da fragment ion can be explained by the loss of PTC, thus representing the singly positive charged ion of Xa. The fragment ion of 102.05498 Da can be explained by simultaneous

| Amino acid | C. australis | A. coffeaeformis |
|------------|-------------|-----------------|
| Gly        | 18.8        | 6.4             |
| Ala        | 5.4         | 5.6             |
| Val        | 2.7         | 4.3             |
| Leu        | 1.6         | 4.0             |
| Ile        | 1.5         | 2.5             |
| Pro        | 1.8         | 1.3             |
| Met        | 2.9         | 1.4             |
| Phe        | 1.3         | 2.2             |
| Tyr        | 1.8         | 3.5             |
| Trp        | t.a.        | t.a.            |
| Ser        | 22.4        | 9.7             |
| Thr        | 12.1        | 5.1             |
| Cys        | 1.0         | t.a.            |
| Hyp        | n.d.        | t.a.            |
| DiHyp (Xa) | 17.0        | 3.1             |
| Lys        | 0.7         | 2.0             |
| Arg        | 0.7         | 27.4            |
| His        | t.a.        | t.a.            |
| Asx^a      | 4.8         | 6.8             |
| Glx^a      | 4.4         | 14.7            |

Table 2. Amino acid composition of the adhesive material from *C. australis* and *A. coffeaeformis*.

^aDuring acid hydrolysis Asn and Gln are converted to Asp and Glu. Asx = Asp+Asp; Glx = Glu+Glu.

t.a. = trace amounts, n.d. = not detected. The non-standard amino acids Xb, Xc and Xd have not been quantified as they are present only in small amounts and their chemical structures have remained unidentified.
loss of PTC and CH$_2$O$_2$. In CID fragmentation of amino acids the loss of CH$_2$O$_2$ results from elimination of the α-carboxylic acid group as formic acid (Koenig & Fales 2011). Therefore, X$_a$ exhibits the general structure of an α-amino acid with the atomic composition C$_{5}$H$_{9}$NO$_{4}$. These characteristics perfectly match the structure of 3,4-dihydroxy-L-proline (Dihyp), which is a very rare natural amino acid, but has previously been identified in silica-associated proteins from diatom (Nakajima & Volcani 1969; Poulsen & Kröger 2004) and mussel adhesion proteins (Taylor et al. 1994). Indeed, in FTMS analysis the PTC-derivative of synthetic Dihyp exhibited the same fragmentation pattern as PTC-X$_a$ (see Figure S5). To further investigate whether X$_a$ is identical to Dihyp, two synthetic stereoisomers of Dihyp (courtesy of C. Taylor, Louisiana State University, Baton Rouge, LA, USA), the 2,3-trans-3,4-cis isomer (cisDihyp) (Weir & Taylor 1999) and the 2,3-cis-3,4-trans isomer (transDihyp) (Taylor et al. 2005), were derivatized with PITC and analyzed via RP-HPLC. The retention time of PTC-transDihyp perfectly matched the retention time of PTC-X$_a$ whereas PTC-cisDihyp exhibited the same retention time as glutamic acid (Figure S6). When the underivatized amino acids were separated by high pressure anion exchange chromatography (HPAEC) X$_a$ and transDihyp still exhibited identical retention times, whereas the retention times of cisDihyp and glutamic acid were markedly different (Figure 4). This confirmed the identity of X$_a$ with transDihyp and revealed that cisDihyp is not a component of the adhesive material from either diatom species (Figure 4). Altogether, the data from mass spectrometry and chromatography analyses demonstrate that the adhesive materials from C. australis and A. coffeaeformis contain 3,4-dihydroxy-L-proline, which likely represents the 2,3-cis-3,4-trans isomer (note: there are four possible more stereoisomers of 3,4-dihydroxy-L-proline).

Previously, the tyrosine derivative Dopa has been identified in a number of proteins that are involved in underwater adhesion, including those from marine mussels (Lee et al. 2011) and sand-castle worms (Phragmatopoma sp.) (Waite et al. 1992). The methods for amino acid analyses described above did not allow the detection of Dopa as they were not compatible with the high oxygen sensitivity of Dopa at alkaline pH. To investigate the presence of Dopa, the acid hydrolysates of the isolated diatom adhesive materials were directly subjected to amino acid analysis by ion exchange chromatography at acidic pH and post-column derivatization with ninhydrin. This method has previously been successfully employed for sensitive detection of Dopa from mussel adhesion proteins (Waite 1991). Dopa could not be detected in the isolated adhesive material from either diatom species (Table S5). When free Dopa was added to the adhesive material before hydrolysis 75–100% of the added Dopa was recovered in the amino acid analysis, demonstrating the validity of the analytical method. As the presence of Dopa has not yet been investigated for any diatom, amino acid analysis was performed of whole cell hydrolysates of C. australis and A. coffeaeformis. Dopa was not detected in either of the two species. When Dopa was added to the cell pellets before hydrolysis, 86–100% of the added Dopa was recovered, demonstrating that the identification of Dopa in the complex diatom hydrolysates is feasible. These data demonstrate that Dopa is absent from the isolated diatom adhesive material. The presence of Dopa-derived cross-linking products (eg due to reaction of Dopa-quinone with amino or thiol groups) in the diatom adhesive materials is highly unlikely, as C. australis and A. coffeaeformis cells appear to be incapable of biosynthesis of Dopa and Dopa containing proteins.

**Discussion**

The present work has identified for the first time the carbohydrate and amino acid composition of underwater adhesives from diatoms. The absence of Dopa indicates that diatom adhesion, in contrast to surface adhesion of the mussel byssal footpad, is not mediated by the direct interaction of Dopa residues with the substratum surface. The underwater adhesives of barnacles and caddisfly larvae also lack Dopa (Stewart, Ransom, et al. 2011; Kamino 2013). The Dopa-free adhesion proteins are generally rich in hydrophilic and charged amino acid residues, which may act synergistically in displacing water, ions, and low-affinity ligands from the substratum surface, thus allowing the protein to bind non-covalently yet strongly to the surface using polyvalent interactions and hydrogen bonding (Stewart, Wang, et al. 2011). As demonstrated in the present work, diatom adhesive proteins are also highly hydrophilic. They are composed of ~75% polar amino acids and also contain 2,3-cis-3,4-trans-dihydroxy-L-proline (transDihyp), a rare amino acid.
acid, whose function has so far remained unknown (Nakajima & Volcani 1969; Taylor et al. 1994; Poulersen & Kröger 2004). The two hydroxyl groups of trans-Dihypro contribute to the hydophilicity of the adhesive material and its pyrrolidine backbone may promote random coil conformation of the polypeptide components, which is believed to be advantageous for surface adhesion (Stewart, Wang, et al. 2011).

The preponderance of carbohydrates and the presence of high amounts of uronic acids are features that diatom adhesives share with exopolysaccharides of bacterial biofilms (Sutherland 2001). Carbohydrates are also present in the adhesives secreted by the sea star, Astoria rubens (Hennebert et al. 2011), in the green mussel Perna viridis (Ohkawa et al. 2004), in the limpet adhesive mucus (Smith et al. 1999) and in the zoospore adhesive of the green alga Ulva linza (formerly Enteromorpha linza) (Stanley et al. 1999). The carbohydrate moieties further increase the hydophilicity of the adhesive material, and in the case of uronic acids also introduce additional carboxylate groups. The latter may promote cohesion of the adhesive polymers through ionic crosslinking via polyvalent metal ions (eg Ca^{2+}, Fe^{2+}), and through direct ionic interactions with amino groups of the polypeptide backbone. It has been proposed that strong cohesion between polyelectrolyte components (ie dehydration by complex coacervation) is an essential prerequisite for surface adhesion (Stewart, Wang, et al. 2011). The presence of high amounts of arginine residues in the adhesion proteins of *A. coffeaeformis* is consistent with this hypothesis, but the adhesive proteins of *C. australis* exhibit only a low abundance of amino-group bearing amino acids. However, it is possible that the adhesive proteins from *C. australis* contain non-standard cationic amino acid residues that have not been detected in the present analyses.

The differences in the chemical composition of the adhesive material from *C. australis* and *A. coffeaeformis* reported in the present study are consistent with previously reported differences in their adhesion strengths (Holland et al. 2004), motility rates (Holland et al. 2004; Molino et al. 2006) and viscoelastic properties (Molino et al. 2006, 2008). Additionally, structural features of the cell wall can also contribute to the adhesive strength as was recently demonstrated by Kopanska et al. (2014) using acid-cleaned diatom cell walls. They demonstrated that cell walls from *A. coffeaeformis* exhibited higher adhesion strengths compared to *C. australis* cell walls. The higher adhesion strength of *A. coffeaeformis* cell walls was attributed to the cells’ lower aspect ratio and higher surface contact area with the substratum, resulting in a reduced hydrodynamic force that is exerted on the cell walls (Kopanska et al. 2014).

The insolubility of the *C. australis* and *A. coffeaeformis* adhesives suggests curing through ionic and/or covalent cross-linking, which is a common feature in underwater adhesives (Stewart, Wang, et al. 2011). Indeed, AFM measurements of the adhesive trails secreted by *Pinnularia* sp. revealed that the trails are initially soft and sticky, and after 2–6 days they become more stable suggesting that they are curing with time (Higgins et al. 2000). Chiovitti et al. (2008) proposed that the sulfated, high-molecular-mass glycoproteins in adhesive pads from the centric diatom *Toxarium undulatum* are cross-linked by calcium and magnesium ions. Quartz crystal microbalance studies revealed that the viscoelastic properties of the adhesives produced by *C. australis* and *A. coffeaeformis* remained unchanged during the measurement period (20 h), which indicates that the cross-linking status of the adhesive material does not change during the first 20 h of secretion. The insolubility of the adhesive material isolated in the present study, which had been isolated within 24 h after secretion from the cells, may thus be explained by immediate cross-linking of the adhesive macromolecules as they emanate from the cell and become deposited on the substratum. Finding methods to dissolve the isolated diatom adhesive material with minimal destruction of the biopolymer backbones will be challenging. However, this is an essential prerequisite for future work on characterizing the primary structures of the carbohydrate and protein components and elucidating their adhesion mechanisms.

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