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Original Article

Deciphering the molecular mechanisms underlying sea urchin reversible adhesion: A quantitative proteomics approach

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ABSTRACT

Marine bioadhesives have unmatched performances in wet environments, being an inspiration for biomedical applications. In sea urchins specialized adhesive organs, tube feet, mediate reversible adhesion, being composed by a disc, producing adhesive and de-adhesive secretions, and a motile stem. After tube foot detachment, the secreted adhesive remains bound to the substratum as a footprint. Sea urchin adhesive is composed by proteins and sugars, but so far only one protein, *Nectin*, was shown to be over-expressed as a transcript in tube feet discs, suggesting its involvement in sea urchin adhesion. Here we use high-resolution quantitative mass-spectrometry to perform the first study combining the analysis of the differential proteome of an adhesive organ, with the proteome of its secreted adhesive. This strategy allowed us to identify 163 highly over-expressed disc proteins, specifically involved in sea urchin reversible adhesion; to find that 70% of the secreted adhesive components fall within five protein groups, involved in exocytosis and microbial protection; and to provide evidences that *Nectin* is not only highly expressed in tube feet discs but is an actual component of the adhesive. These results give an unprecedented insight into the molecular mechanisms underlying sea urchin adhesion, and opening new doors to develop wet-reliable, reversible, and ecological biomimetic adhesives.

Significance: Sea urchins attach strongly but in a reversible manner to substratum, being a valuable source of inspiration for industrial and biomedical applications. Yet, the molecular mechanisms governing reversible adhesion are still poorly studied delaying the engineering of biomimetic adhesives. We used the latest mass spectrometry techniques to analyze the differential proteome of an adhesive organ and the proteome of its secreted adhesive, allowing us to uncover the key players in sea urchin reversible adhesion. We demonstrate, that *Nectin*, a protein previously pointed out as potentially involved in sea urchin adhesion, is not only highly expressed in tube feet discs, but is a genuine component of the secreted adhesive.

1. Introduction

Adhesives found in nature perform in ways that man-made products simply cannot match. Some are reversible, others are very effective underwater and many are universal in their performance to substrata of varying composition and structure. Yet, only a very limited number of model systems have inspired novel biomimetic adhesives, including the well-known gecko foot for dry adhesion and mussel glue for wet adhesion [1]. In order to speed up the engineering of innovative adhesives, it is essential to understand better how biological adhesives function, including their mode of action, their basic components, building principles and function-specific adaptations selected by evolution. Sea urchin reversible adhesion is no exception. Although in the last decade a significant effort was made to answer many questions regarding morphology and biomechanical properties of sea urchin adhesive organs, and the

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molecular mechanisms underlying sea urchin adhesion reversibility remain largely a mystery [2]. Nevertheless, being water-resistant, effective on natural and man-made substrata and reversible, sea urchin adhesives have great potential to inspire the development of new biomimetic surgical and dental adhesives, cell/tissue immobilizing agents but also cell/molecule displacers and antifouling coatings.

In sea urchins, adhesion takes place at the level of a multitude of small appendages, adoral tube feet, and involves the secretion of an adhesive between these specialized organs and the substratum (Fig. 1A). Tube feet are used in locomotion and attachment, being extremely well designed for reversible adhesion. They are composed of an enlarged and flattened apical disc that makes contact with and adheres to the substratum, and an extensible tether, the stem, allowing the tube foot to lengthen, flex and retract (Fig. 1B) [3]. Morphological studies have shown that when the substratum is suitable, the disc reorients its apical surface to be parallel to the substratum, makes contact and releases the content of adhesive granules contained inside specialized secretory cells, thus initiating the attachment process. Detachment of tube foot is accomplished by the release of de-adhesive granules content by a second type of secretory cells, breaking the bonds established between the adhesive and the disc, thus leaving a thin layer of adhesive material (footprint) strongly attached to the substratum (Fig. 1D) [2]. Yet, sea urchin tube feet do not contain exclusively these secretory cells, but present a complex histological structure related with their adhesive function, being composed of an inner myomesothelium, a connective tissue layer, a nerve plexus and an outer epidermis covered externally by a well-developed, multilayered glycocalyx, the cuticle (Fig. 1C) [3].

Although numerous individuals are necessary to obtain a sufficient amount of sea urchin adhesive material, earlier proteomic studies successfully identified proteins extracted from the sea urchin *Paracentrotus lividus* secreted adhesive [4]. The water content of the obtained adhesive was not measured but, in terms of dry weight, it is mainly made up of proteins (6.4%), lipids (2.5%), carbohydrates (1.2%) and a large inorganic fraction (45.5%) [4]. The protein fraction of *P. lividus* adhesive was further characterized in terms of amino acid composition, highlighting a bias towards 6 amino acids (glycine, alanine, valine, serine, threonine, and asparagine/aspartic acid), together with higher levels of proline (6.8%) and half-cysteine (2.6%) than the average eukaryotic proteins [4]. These traits are common to many marine adhesives and are pointed out as key factors for their high adhesive strength, cohesion and insolubility. *P. lividus* adhesive insolubility was partially overcome using strong denaturing and reducing conditions, from which 13 proteins could be extracted, and 6 were identified by mass spectrometry as alpha and beta tubulin, actin, and the histones H2A, H2B, H3 and H4 [4].

To bypass the challenge of solubilizing the secreted adhesive, a subsequent study performed protein extraction on dissected adhesive tube feet discs, a source of soluble adhesive and de-adhesive precursors [5]. The adhesive disc proteome was shown to contain 328 non-redundant proteins, of which only 2% were putative adhesive proteins [5]. Among these was *Nectin*, a *P. lividus* cell adhesion protein secreted by eggs and embryos [6,7], never before reported in the adult adhesive organs. Recent research showed that adult tube feet express two mRNA *Nectin* variants (GenBank AJ578435 and KT351732) that are over-expressed (2.5-fold) in the tube feet disc relatively to the stem, their expression being localized in the disc adhesive secretory cells and cuticle, thus suggesting an involvement in sea urchin adhesion [8]. Besides *Nectin*, only one more *P. lividus* protein was pointed out as putatively adhesive — *Toposome* [5], which is a modified calcium-binding iron-less transferrin also secreted in eggs and embryos [9,10]. In terms of putative de-adhesive proteins, surprisingly, no proteases or glycosylases that could trigger sea urchin tube foot de-adhesion by degradation of the secreted adhesive components were identified until now [5].

Sea stars are sea urchin's close relatives, being also echinoderms and attaching with a temporary adhesive secretion produced by their tube feet. Both adhesives have a similar biochemical composition; contain high amounts of small side-chain and charged/polar amino acids, probably for high cohesive strength and interactions with the substratum, respectively [4,11]; and high amounts of cysteine most certainly responsible for their insolubility [4,11]. Although putative adhesive and de-adhesive proteins have been extracted from sea star and sea urchin adhesive organs and adhesive secretions [4,5,12,13], up to now only one protein, sea star footprint protein 1 (Sfp1), was unequivocally assigned as a constituent of the sea star adhesive [14]. However, upon secretion, Sfp1 forms a structural scaffold and thus appears to provide sea star adhesive with cohesiveness rather than adhesive properties [14].

Therefore, although promising, the available molecular information on echinoderms reversible adhesion remains scarce, indicating that other approaches are needed. In this study we used a label-free quantitative proteomic approach coupled with high-resolution mass-spectrometry to perform the first differential proteome of an adhesive organ, comparing protein expression levels in tube foot adhesive part (disc) *versus* non-adhesive part (stem), revealing the key proteins involved in sea urchin reversible adhesion. We also profiled the proteome of *P. lividus* secreted adhesive disclosing its major components, strongly supported by western-blot and immunohistochemistry evidences of the obtained expression patterns and protein identifications.

2. Material and methods

2.1. Sample preparation

Sea urchins from the species *P. lividus* (Lamark 1816) were collected at low tide on the west coast of Portugal (Estoril, Portugal). After collection, the animals were transported to “Vasco da Gama Aquarium” (Dafundo, Portugal) and kept in open-circuit tanks at a temperature of 15 °C and salinity of 33‰. Then, sea urchins were placed in small plastic aquariums (3 L) filled with seawater, covered internally with removable glass plates to which animals were allowed to attach and then forced to

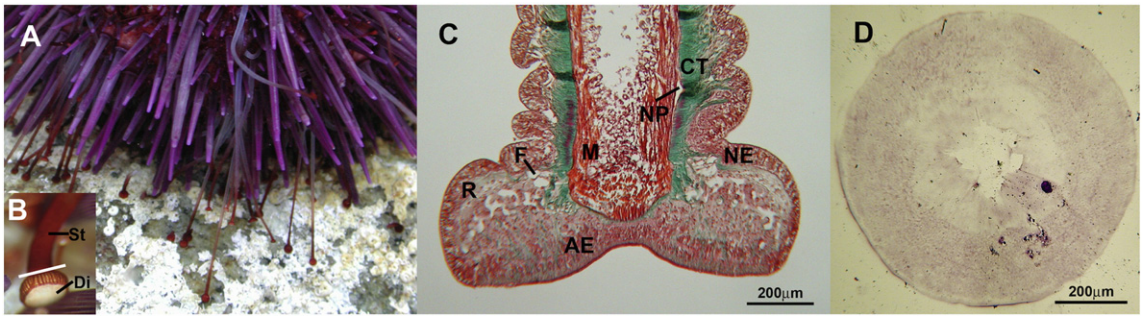

Fig. 1. Sea urchin *Paracentrotus lividus* attaching to a rock with its adoral tube feet. (A) Enlarged view of a tube foot (B) composed by a disc (Di) and a stem (St). Histological structure of an adoral tube foot (C) stained with Masson's trichrome showing the disc adhesive epidermis (AE) with its ossicles, the frame (F) and the rosette (R), the stem non-adhesive epidermis (NE), the nerve plexus (NP), the connective tissue (CT) and the muscle (M). Adhesive footprint left on the substratum after tube foot detachment (D) stained with 0.1% Crystal Violet.