Adhesion molecules in two species of echinoderms *Marthasterias glacialis* and *Holothuria forskali*

Dissertation
zur Erlangung des Grades
Doktor der Naturwissenschaften

Am Fachbereich Biologie
der Johannes Gutenberg-Universität
in Mainz

Malgorzata Baranowska
geb. in Jarocin (Polen)

Mainz, 2008

Index

1. Introduction 1
1.1. Background to biological adhesion 1
1.2. Review of echinoderm adhesive system 2
1.2.1 Tube feet 3
1.2.2 Larval adhesive 5
1.2.3 Cuvierian tubules 6
1.2.3.1 Measurement of adhesion of Cuvierian tubules 7
1.3. Comparison with other invertebrates 8
1.3.1 Permanent adhesion 8
1.3.1.1 Mussels 9
1.3.1.2 Barnacles 10
1.3.2 Temporary adhesion 10
1.3.2.1 Hermans hypothesis 11
1.4 Non secretory adhesive systems in organisms 12
1.5 Application of echinoderms bio-adhesives 12
1.5.1 Water resistant adhesive 13
1.5.2 Antifouling strategies 14
1.6 Overview of studied animals 14
1.6.1 Asteroidea 14
1.6.2 Holothuroidea 16
2. Aim of the study 17
3. Materials… 18
3.1 Chemicals 18
3.2 Equipment 21
3.3 Antibodies 23
3.4 Markers 23
3.5 Kits… 23
3.6 Experimental animals 24
3.6.1 Starfish 24

3.6.2 Sea cucumber 24
4. Methods........ 25
4.1 Methods used to isolate adhesive material 25
4.1.1 Collection of adhesive material 25
4.1.1.1 Starfish 25
4.1.1.2 Sea cucumber 25
4.1.2 Extraction of adhesive material 26
4.1.2.1 Starfish 26
4.1.2.2 Sea cucumber 27
4.1.3 Purification of crude extract for SDS PAGE analysis 28
4.1.3.1 PAGE prep® Advance Kit 28
4.1.3.2 Ethanol precipitation 28
4.1.3.2 TCA/Acetone precipitation 29
4.1.3.3 ReadyPrep™ 2-D Cleanup Kit 30
4.1.4 SDS Polyacrylomide gel electrophoresis (PAGE) 30
4.1.4.1 Seminative PAGE 32
4.1.5 Stains for SDS PAGE 33
4.1.5.1 Gel Code Blue Stain 33
4.1.5.2 Glycoprotein stain 34
4.1.5.3 Silver stain 34
4.1.5.4 Imperial Stain 35
4.1.5.5 Sypro Ruby 36
4.1.6 Antibody production 36
4.1.7 Western Blot 36
4.1.8 Neutralisation of antibody 38
4.1.9 Immunoprecipitation 38
4.1.10 Electro-elution 39
4.1.11 Glass Beads 41
4.1.12 Gel Filtration 42
4.1.13 Digestion 44
4.1.14 HPLC 45

4.2 Methods used for characterisation of adhesive material 48
4.2.1 Protein concentration assays 48
4.2.1.1 Bradford Assay 48
4.2.1.2 Lowry Assay 48
4.2.2 Measurement of adhesion 50
4.2.3 Preparation sections on slides 56
4.2.4 Immunochemistry 56
4.2.5 Stain Slides 57
4.2.5.1 Haematoxylin & Eosin 57
4.2.5.2 Alcian blue, PAS 58
4.2.5.3 Coomassie Blue 58
4.2.5.4 Alcian blue, Ponceau, Acid fuchsine 59
4.2.5.5 Casson's Trichrome 59
4.2.5.6 Methylene Blue – Azure B 60
4.2.5.7 Haematoxylin & Oil Red O .... .60
4.2.5.8 Toulidyne blue 61
4.2.6 AFM 61
4.2.7 Wyko 63
4.2.8 Transmission electron microscopy 64
5. Results…... 65
5.1 Sea cucumber *Holothuria forskali* 65
5.1.1 Isolation and identification of the adhesive protein from the Cuvierian tubules 65
5.1.1.1 Extraction of Cuvierian tubules 65
5.1.1.2 Identification of adhesive protein 67
5.1.1.3 Isolation of identified protein 68
5.1.1.3.1 Electro-elution 68
5.1.1.3.1.1 Neutralisation of antibody 69
5.1.1.3.2 Immunoprecipitation 70
5.1.1.3.3 Gel filtration 71
5.1.1.3.4 Glass beads 72

5.1.2 Characterisation of adhesive protein in Cuvierian tubules 73
5.1.2.1 Histological identification 73
5.1.2.2 Immunostaining.. 75
5.1.2.3 Immunocytochemistry.. 77
5.1.2.4 Measurement of adhesion.. 77
5.1.2.5 Labelling adhesive protein in mussel threads using
antibody from CT 87
5.2 Starfish *Martasterias glacialis* 89
5.2.1 Isolation of the adhesive protein from the tube feet and footprint
of starfish 89
5.2.1.1 Protein extraction 89
5.2.1.2 Purification extract for SDS-PAGE 90
5.2.1.3 SDS-PAGE of the tube feet and footprint extracts 90
5.2.1.4 Purification of adhesive protein using HPLC 93
5.2.2. Characterisation of adhesive protein from the tube feet and footprint
of starfish 95
5.2.2.1 Histology 95
5.2.2.2 AFM images of the footprint.. 97
5.2.2.3 Wyko images of the footprint... 99
5.2.2.4 Characterisation of adhesive protein from starfish using antibody
against adhesive protein from the Cuvierian tubules 101
6. Discussion. 103
7. Summary... 119
8. Summary (germ.) 121
9. References 123
10. List of abbreviations 135

1. Introduction

Marine organisms posses a wide range of mechanisms which allow them to move, attach, burrow and feed. In many invertebrates these activities are facilitated by adhesive mechanisms though these are generally, poorly understood. In this study the attachment of two species of echinoderms and the specialised structures used for this function were investigated. The main task in this research was to recognise in these organisms the molecules responsible for adhesion and further their isolation and characterisation.

Anatomical, histological and physiological investigations of organs used for adhesion have previously been made by many researchers (Nichols, 1961; Kawaguti, 1964; Barker, 1978; VandenSpiegel and Jangoux, 1987; McKenzie, 1987; Flammang, 1996) but isolation of the adhesive molecules has not been studied to date (DeMoor, 2003), largely because of the technical difficulties in achieving this. In this study classical histology methods have been complemented with modern approaches of molecular biology and advanced microscopy.

1.1 Background to biological adhesion

Adhesives are first known to have been used by the Sumerian people, who used natural gums and other plant resin or saps as adhesives. Most early adhesives were animal glues made by rendering animal products such as horse hooves. The Native Americans of the Eastern United States used a mixture of spruce gum and fat as adhesives to add waterproof seams in their birch bark canoes (Warren and Gidmark, 2001). In Babylonia statues were assembling using tar-like glues and in Egypt animal glues were utilised for furniture, ivory and papyrus (Nicholson and Shaw, 2000). In Medieval Europe/Eurasia, egg whites were used as a glue to decorate parchments with gold leaves. The first known glue factory was founded in Holland, in the early 1700's. In the 1750s, the British introduced fish glue (Firth, 1969). During next centaury, several other patented materials, such as bones, starch, fish, and casein, were introduced. Modern glues have improved

beyond recognition from the early methods. Such improvements are noticeable in the flexibility, toughness, curing rate, temperature, and chemical resistance of modern glues.

In recent years research has focused on developing water resistant adhesives from marine organisms like mussels, barnacles and echinoderms. Echinoderm adhesion is poorly understood and the task in this study is to add to our knowledge about their adhesives.

1.2 Review of echinoderm adhesive systems

Echinoderms (Phylum Echinodermata, from the Greek for spiny skin) are exclusively marine animals which are can be found in all depths in the ocean. The first definitive members of the phylum appeared near the start of the Cambrian period, and the phylum contains about 7000 living species, making it the second largest grouping of deuterostomes, after the chordates. They are the largest phylum without freshwater or terrestrial representatives.

Echinoderms are classified into five easily recognised classes:

Sea star or starfish (Asteroidea)

Brittle stars, basket stars, serpent stars (Ophiuroidea)

Sea urchins, heart urchins and sand dollars (Echinoidea)

Holothurians or sea cucumbers (Holothuroidea)

Feather stars and sea lilies (Crinoidea)

Adhesion is a way of life in echinoderms. All the species from this phylum use adhesive secretions for various functions (Flammang *et al.*, 2005). They use different adhesive systems for various functions, the most studied being:

1) tube feet or podia which are used for vital function
2) larval adhesive use for settlement during metamorphosis
3) Cuvierian tubules use for defence

1.2.1 Tube feet or podia

Echinoderms are benthic organisms and they have activities and adaptations which are correlated with benthic existence. For these activities, like attachment to substratum, locomotion, handling and burrowing echinoderms use tube feet. These are external appendages of the ambulacral system and also probably the most advanced hydraulic organs in the animal kingdom (Smith, 1937; Nichols, 1966; Flammang, 1996). In post-metamorphic echinoderms, specialized organs – the podia or tube feet – have always have been found to produce adhesive secretions. The histological structure of the tube feet is similar for all echinoderm species.

Their tissue consists of four layers: an inner endothelium and an outer epithelium, with a connective tissue layer and a nerve plexus (Kawaguti, 1964; Nichols, 1966; Florey and Cahill, 1977; Flammang and Jangoux, 1992). Adhesion in starfish is the function of the tube feet or podia and their histological structure is typical for the echinoderms. On the tube foot tip there are tissue layers specialized in adhesion and sensory perception: the connective tissue layer and the nerve plexus are thickened and the epidermis is differentiated into a well – developed sensory – secretory epithelium. Light microscopy studies have revealed specialised grandular areas, in the epidermis of podia. These areas are always located at the level functionally important parts of the podia, such as the disc surface responsible for adhesion (Smith, 1937; Nichols, 1959; Perpeet and Jangoux, 1973; McKenzie, 1987).

Tube feet have diversified into wide variety of morphotypes, which were classified by Flammang (1996) into disc-ending, penicillate, knob-ending, lamellate, ramified and digitate. Knob-ending, disc-ending and reinforced disc-ending have been most studied.

Knob-ending tube feet

This type of foot is made up of a basal cylindrical stem ending distally with a pointed knob (Fig. 1 A). The water-vascular lumen extends into the knob where it tapers off to a point. All tissue layers are thicker in the knob than in the stem, except the mesothelium.

The adhesive epidermis is composed of granule-filled secretory cells belong to a tall

columnar epithelium. The nervous tissue consists of a nerve ring on the proximal side of the knob and a thick basiepithelial nerve plexus underlying the adhesive epidermis. Both layers lie on basal lamina that is backed by a thick layer of loose connective tissue. This type of podia was found in echinoids (Nichols, 1959; Smith, 1980) also in asteroids, there are knob-ending podia at the tip of each arm (Sloan and Campbell, 1982). Aspidochirote holothuroids posses knob-ending podia too, which are so-called papillae (McKenzie, 1987; Vanden Spiegel *et al.,* 1993; 1995).

Simple disc-ending tube feet

Simple disc-ending tube feet consist of a basal cylindrical stem with an apical extremity that is enlarged and flatted to form the so-called disc (Fig. 1 B).

The disc has the same basic structure as the knob of knob-ending tube feet: it encloses the distal extremity of the water-vascular lumen and its epidermis, nerve plexus and connective tissue layer are thickened compared to their equivalents in the stem.

Most podia of regular echinoids, asteroids and dendrochirote and aspidochirote holothuroids end with disc which are involved in locomotion and attachment (Smith, 1937, 1947; Nichols, 1961 and Smith, 1978)

Reinforced disc-ending tube feet

Reinforced disc-ending tube feet have more or less the same external morphology as simple disc-ending tube feet: they comprise a basal cylindrical stem topped by a flattened disc (Fig.1 C). However, the diameter of their disc never greatly exceeds that of the stem.

The histological organization of the reinforced tube feet is close to that of simple disc ending tube feet except that their lumen does not extend into the disc margin and that their disc connective tissue layer sends numerous bundles of collagen fibres distally that insinuate themselves within the adhesive epidermis (Santos, 2005).

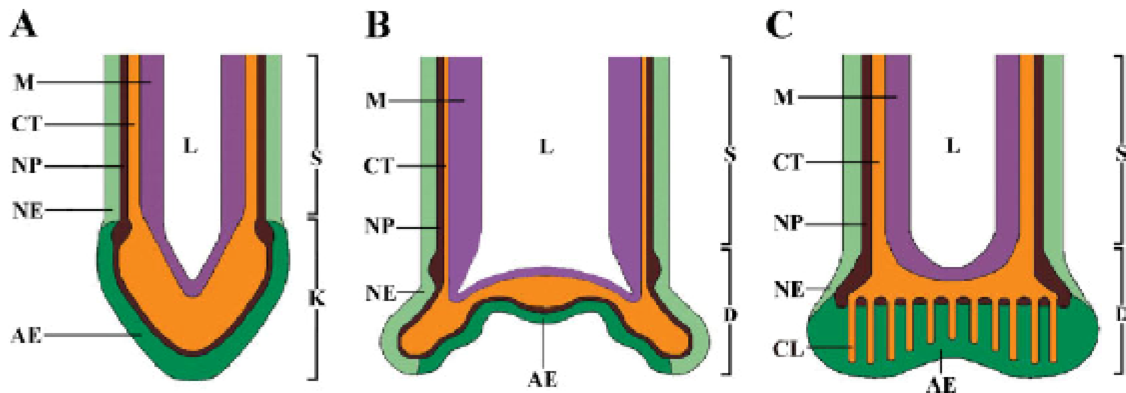


Fig. 1 Schematic drawings of the three tube foot morphotypes observed in asteroids (not to scale). **A** – Knob-ending tube foot, **B** – Simple disc-ending tube foot, **C** – Reinforced disc-ending tube foot, **AE** – adhesive epidermis, **CL** – connective tissue radial lamellae, **CT** – connective tissue layer, **D** – disc-shaped tube foot tip, **K** –knob-shaped tube foot tip, **L** – water-vascular lumen, **M** – myomesothelium, **NE** – nonadhesive epidermis, **NP** – nerve plexus, **S** – tube foot stem (Santos, 2005).

Crinoid podia are different than in other echinoderms because they arise and function in groups of three. Morphology of the podial triplets was described by Chadwick, 1907 and follow by Reichensperger, 1908; Gislen, 1924 and Nichols, 1960. Each triplet member in this species has a behaviour related to its role in suspension feeding (Byrne and Fontaine, 1981). The sticky surface of crinoid podia was noted by Reichensperger, 1908; Gislen, 1924. The adhesive structures of the tube feet are located in papillae (McKenzie, 1992).

1.2.2 Larval adhesive

In almost all echinoderms, metamorphosis transforms a bilaterally symmetrical and pelagic larva into a radially symmetrical and benthic postmetamorphic individual. Perimetamorphic period is the time when settlement take place (Gosselin and Jangoux, 1998; Haesaerts *et al.,* 2003) but either before or after the metamorphic stage according to the class considered (Strathmann, 1978). In both cases, adhesive organs attach the competent larva to the substratum during settlement. In most of echinoderms classes, these organs are the tube feet, they are similar in structure and function to the tube feet of adults (Cameron and Fankboner, 1984; Flammang *et al.,* 1998b).

I.2.3 Cuvierian tubules

Cuvierian tubules are peculiar organs found in several species of holothuroids (sea cucumbers), all belonging exclusively to the family Holothuriidae (Smiley, 1994; Lawrence, 2001). There are two main types of Cuvierian tubules: smooth and lobulated (Lawrence, 2001). The smooth tubules occur in holothuroids of the genera *Bohadschia*, *Holothuria* and *Pearsonothuria* are expelled as sticky white threads that function in defence against predators (VandenSpiegel and Jangoux, 1987; Hamel and Mercier, 2000). The lobulated tubules occurring in aspidichorote holothuroids of the genus *Actinopyga* are never expelled and are not sticky (VandenSpiegel and Jangoux, 1993). The function of these tubules has not been explained yet (Lawrence, 2001). In aspidichirotes that employ Cuvierian tubules defensively, the expelled tubules lengthen, become sticky and rapidly immobilize most organisms with which they come in contact (Jourdan, 1883; Mines, 1912; Crozier, 1915). The tubules consist of: from the inside to the outside, an inner epithelium surrounding the narrow lumen, a thick connective tissue layer and a mesothelium lining the surface of the tubule that is exposed to the coelomic cavity.

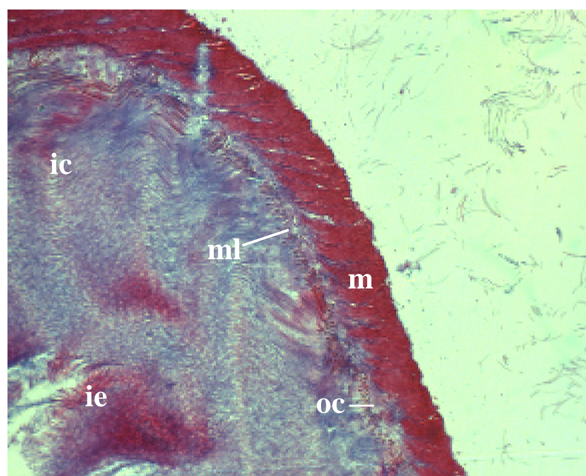
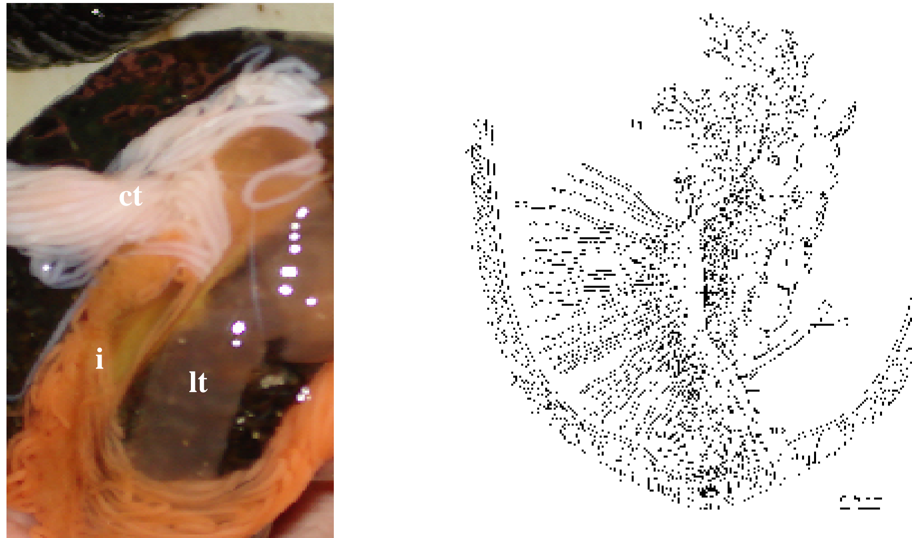


Fig. 2 Longitudinal section through the wall of a tubule (**ic** – indicates inner connective tissue layer, **ie** – inner epithelium, **m** – mesothelium, **ml** – muscle layer, **oc** – outer connective tissue layer)

The inner epithelium consists of cells enclosing large heterogeneous spherules composed of preiniotc and glucidic fractions (Guislain, 1953; VandenSpiegel and Jangoux, 1987). The connective tissue forms about 90% of the thickness of Cuvierian tubules. Two areas

can be recognised, a thin outer area between the mesothelium and the muscle fibres and a thick inner area located between the muscle fibres and the epithelium (VandenSpiegel and Jangoux, 1987). Tubules have a pseudostratified mesothelium made of two cell layers, an upper layer of adluminal cells and a lower layer of granular cells. Attachment of the Cuvierian tubules is present on Fig. 3.



VandenSpiegel, Jangoux, 1987

Fig. 3 Holothuria *forskali*. Attachment area of the Cuvierian tubules, **a** – anus; **c** – cloaca; **cc** – cuvierian chamber; **cr** – cloaeal rupture; **ct** – Cuverian tubules; **dm** – digestive mesentery; **i** – intestine; **lt** – left respiratory tree; **ms** – mesenteric strand; **rt** – right respiratory tree

I.2.3.1 Measurement of adhesion of Cuvierian tubules

The measurement of the adhesive strength of Cuvierian tubules and its variation under different conditions may give clues to the mechanism of marine bioadhesion.

The adhesion of tubules is remarkable in several respects. The composition of the adhesive is very different than in other adhesive secretions found in marine invertebrates (DeMoor *et al.,* 2003). Adhesion is achieved in matter of seconds (Zahn *et al.,* 1973). In the early 1970's two studies on the adhesion of *Holothuria forskali* (Müller *et al.,* 1972; and Zahn *et al.,* 1973) were published, which were focused on the influence of various chemical reagents on the adhesive strength of Cuvierian tubules attached to a paraffin wax substratum. This research was follow by Flammang *et al.,* 2002 who measured how

adhesion was influenced by different factors such as temperature and salinity. Müller (1972) used a traction method to measure adhesion. He showed that traction is caused by hydrophobic interactions (i.e. polarized water prefer bonding itself and repels hydrophobes) and can be influenced by proteolytic enzymes to various degrees. Carboxypeptidase A was the most active compound and posses two phase of action. Urea in low concentration doubled the traction, while in high concentration it brought to zero. Only phenylalanine and leucine inhibited the traction, but other amino acid had no influence on adhesion. Optimal pH for traction is corresponding to the sea water value and amount i.e. 8.2. Zahn (1973) measured traction under the influence of phenylalanine, urea, carboxypeptidase and pronase and found out that most of the enzymes inhibited the adhesion.

1. 3 Comparison with other invertebrates

In the aquatic world many organisms are equipped with the systems to allow them to attach to the substratum. These systems can use mechanical attachment and/or chemical attachment. The attachment can be permanent or temporary.

1.3.1 Permanent adhesion

Permanent adhesion in aquatic environments relies on the release of cement for attachment of organisms that are sessile and remain on a single substrate throughout life after initial settlement, usually as larva (Jangoux and Lawrence, 1996). These organisms can attach to the subject with powerful shear forces and examples include attachment by microalagae, macroalgae, mussels, barnacles and tubeworms to rocks and the hulls of ships (e.g. Walker, 1987; Tyler, 1988; Schnurrer and Lehr, 1996; Burzio *et al.,* 1997). Animals which display such permanent attachment are among the best studied. Their adhesive systems comprise a single type of secretory cell or, if more are present, only one cell type is usually involved in adhesion (Walker, 1970; Tamarin *et al.,* 1976; Waite, 1983; Walker, 1987). On non-polar surfaces the adhesive mechanisms such as hydrogen bonding and binding to (or forming bridges via) metal cations may allow higher sticking forces to be achieved (Whittington and Cribb, 2001)

1.3.1.1 Mussels

Mussels like *Mytilus edulis* attach to various surfaces in aqueous conditions using a natural adhesive which is strong and durable. The first adhesive protein to be isolated and identified was Mefp-1. To date, nine adhesive related proteins have been identified. A tenth is implicated but not isolated (Silverman and Roberto, 2008). The identified protein are: Mefp-1,-2,-3,-4,-5; collagens such as precollagen -D, -P and -NG; proximal matrix thread protein and a polyphenol oxidase but there are probably many other proteins involved in adhesion but which have not been recognised (Silverman and Roberto, 2007).

Mussels attach themselves to solid surfaces through a complex array of threads known as byssus, which allow them to adhere in turbulent aquatic environments. The byssus is connected to the animal by rootlike process and to the solid surface by the adhesive plaques (Tamarin *et al*., 1976; Young and Crisp, 1982). The plaque contains an adhesive protein known as polyphenolic protein which is synthesized and secreted by the phenol gland localised in foot of the animal (Burzio *et al.,* 1990). This protein exhibits strong adhesion to a variety of surfaces such as glass, plastics, slate and metal (Waite, 1987). This highly hydroxylated protein contains a number of amino acids, including 3, 4-dihydroxyphenylalanine (DOPA), which is thought to contribute to the crosslinking of the extruded threads and adhesion to the substratum. The DOPA-rich polyphenolic protein secreted by the marine mussel *Mytilus edulis* establishes key chemical linkages in a water-resistant adhesive (Ooka *et al*., 1990).

Silk fibroin

Precollagen-D found in the distal thread of the mussel adhesive threads contain silk-fibroin domain. Silks belong to tensile strength protein fibres which are also found in spider drag-lines (Gosline *et al*., 1999) and silkworms (Bomyx mori) (Chirila *et al.,* 2008). A similar protein has been found in sea urchins (Pearson, 1981). Silk fibroin found in mussel byssus is responsible for the stiffness and strength of the thread (Waite *et al.,* 1998).

1.3.1.2 Barnacles

Barnacles live in a diverse range of temperatures, salinities and hydrodynamic conditions (Cawthorne and Davenport, 1980) and often form aggregations (Crisp and Meadows, 1962; Matsumura *et al.,* 1996). Both, the adults and larvae stage use adhesive secretions. Barnacle cement is a protein complex that is insoluble underwater. The adhesive properties of the barnacle adhesive proteins have been utilized for various dental and medical purposes (Despain *et al.,* 1973). These polyphenolic proteins are non-toxic and highly effective glues (Khandeparker, 2007).

Attachment of barnacle cyprid larvae is made by hardening of a special adhesive, the so-called cyprid cement (Okano *et al.*, 1997). This cement is secreted from a pair of cement glands and transported through a long, narrow duct to be released at the antennular adhesive disc where hardening takes place (Walker, 1971). The cement must have two physical states, a liquid state, with adequate fluidity both inside storage granules and in the cement duct during transportation, and a solid state at the release site (Crip, 1972).

Barnacles are amongst the most common fouling organisms in the marine environment and are of great economic importance (Southward, 1987). The ability to stay attached to ships that travel with high speed gives an idea about the strength of attachment (around 1×10^{5} N/m^2 (Becker, 1993)). Most studies on the adhesion mechanism of this group have been performed on acorn barnacles (Wiegemann, 2004). Barnacle cement is considered to be the most durable and toughest connection in the living aquatic world (Abbott, 1990). The adhesive is a proteinaceous material (> 90% protein), while the remainder consists of carbohydrate, ash and trace amounts of lipid (Walker, 1999).

1.3.2 Temporary adhesion

This type of adhesion allows organisms to attach momentarily to the substratum. Temporary adhesion permits an organism to attach firmly but momentary to a substrate such as sand grains, other sediment, rocks and variety of other abiotic surfaces. Momentary attachment by adhesives is displayed among tiny invertebrates that inhabit interstitial spaces, such as many turbellarians (Tyler, 1976, 1988; Rieger *et al.,* 1991) and gastrtrichs (Tyler and Rieger, 1980). It also includes the mechanism of adhesion identified for the podia (tube feet) of echinoderm (Flammang, 1996).

1.3.2.1 Hermans hypothesis

Hermans (1983) suggested a new concept about adhesive and de-adhesive system: the duo gland system. He provided descriptions of a number of temporary adhesive systems and suggested that these were all duo-gland systems. In Hermans‘ model the duo-glandular adhesive system as is applies to echinoderm tube feet was based on the following concepts:

1) Adhesion of tube feet is initiated by release of basic, proteinaceous secretions that bind the negatively charged tube foot surface to the interacting surface or particle.
2) De-adhesion of tube feet is invoked by carbohydrate dominated (acid mucopolysaccharide = glycosaminoglycans see Fig. 4) secretions out-competing the adhesives for bonds on tube foot surface.

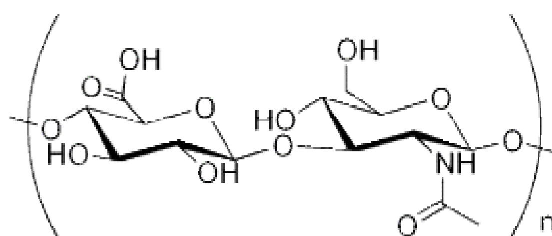


Fig. 4 Hyaluronic acid (example of mucopolysaccharide acid)

3) Proteinaceous adhesive secretions and carbohydrate dominated, de-adhesive secretions are associated with each other in the tube feet to act as duo-glandular adhesive systems.

Hermans' hypothesis has been criticised by McKenzie (1988) who concluded that, while there was ample evidence of adhesive apparatus containing more than one type of secretory cell, there was no direct evidence of a de-adhesive being released onto the tube foot surface. He suggested that de-adhesion was more likely to be a physical event and that more informative ultrastructural and more importantly experimental studies of echinoderm podia were needed.

Another type adhesion which can be distinguished as a type of temporary adhesion is transistory adhesion. This adhesion is characteristic of invertebrates which are moving across a substrate by cilliary gliding (e.g. some tubellarians; see Martin, 1978; Rieger *et al.,* 1991) or muscular wave-like activity (e.g. foot secretions of gastropod molluscs; Grenon and Walker, 1978, 1980; Walker, 1987) and enables adhesion and movement along a substrate simultaneously. Limpets (*Patella*) attach by a thin film of mucus secreted from six types of secretory cells in the foot and this viscous material is left behind as they progress (Grenon and Walker, 1978; Walker, 1987). The pedal disc of sea anemones operates by similar system (Flammang, 1996).

1.4 Non-secretory adhesive systems in organisms

By comparison of insects and other animals it was discovered that non-adhesive attachment systems basically converge to two principal designs: a "hairy" system consisting of finely structured protruding hairs with size ranging from a few hundred nanometres to a few microns, dependent upon the animal species, and a "smooth" system with relatively smooth surface covering a fine tissue microstructure (Niederegger *et al.,* 2002; Gorb *et al.,* 2000). Both systems are able to adapt to the profile of a contacting surface. Geckos, beetles, flies and spiders have adopted the hairy design.

1.5 Application of echinoderm bioadhesives

Bioadhesives are natural polymeric materials that act as glues. Sometimes the term bio-adhesive is used to describe glue formed synthetically from biological monomers such as sugars, or to mean a synthetic material designed to adhere to biological tissue (Peppas

and Sahlin, 1996). Bioadhesives are composed from various substances but the main components are proteins and carbohydrates. Proteins such as gelatine and carbohydrates such as starch have been used as general-purpose glues by humans from pre-historic times. Highly effective adhesives found in natural world are currently under investigation but are not yet in widespread commercial use. For example, bioadhesives secreted by microbes and by marine molluscs and crustaceans are being researched with a view to biomimicry (Smith and Callow, 2006). Biomimicry (from bios, meaning life, and mimesis, meaning to imitate) studies nature, its models, systems, processes and elements and then imitates or takes creative inspiration from them to solve human problems (Benyus *et al.,* 2002).

Bioadhesives are of commercial interest companies because they can be biocompatible, making them useful in biomedical applications involving skin or other body tissue. The fact that some work in wet environments and under water, while others can stick to low surface energy/non-polar surfaces like plastics is very useful for technological applications. In recent years, the synthetic adhesive industry has been impacted by environmental concerns and healthy/safety issues relating to hazardous ingredients, volatile organic compound emissions, and difficulties in recycling or remediation adhesives derived from petrochemical feedstock. Rising oil prices may also stimulate commercial interest in biological alternatives to synthetic adhesives.

The are two main fields of applied research involving bioadhesives the design of water-resistant adhesives and the development of new antifouling strategies (Flammang *et al.,* 2005).

1.5.1 Water resistant adhesive

Biomimetic materials inspired by marine adhesives are therefore sorely needed for applications in wet environments, where problems involve finding the adhesives with enough strength to hold the bond but not to have any adverse environmental impacts. Such materials could be used in underwater construction, but the most important applications are certainly to be found in the biomedical field (Strausberg and Link, 1990; Peppas and Langer, 1994; Taylor and Waite, 1997). Surgical or topical reconnection of

severed tissues is essential for restoration of their structure and function. The most widely used methods for joining tissues focus on mechanical fasteners such as sutures and staples. Surgical adhesives, however, provide attractive alternatives to mechanical fastening (Strausberg and Link, 1990; Albala 2003; Ninan *et al.*, 2003; Singer and Thode, 2004). Dentistry is another field in which there is current demand for non-toxic bioadhesives able to form durable adhesive bonds in the aqueous environment of the mouth (Peppas and Langer, 1994; Burzio *et al.*, 1997).

1.5.2 Antifouling strategies

Biofouling is one of the big problems currently in marine technology. Materials submerged in seawater experience a series of discrete physical, chemical and biological events, which result in the formation of a complex layer of attached organisms known as biofouling (Abarzua and Jakubowski, 1995; Callow and Callow, 2002). Fouling control therefore increasingly a problem of managing adhesion and molecular understanding of how marine bioadhesives work should open up new technologies intended to specifically intervene in organism attachment (Taylor and Waite, 1997; Callow and Callow, 2002).

1.6 Overview of studied animals

The two classes of echinoderm phylum were used for research in this thesis (Asteroidea and Holothuroidea).

1.6.1 Asteroidea (Starfish)

Starfish normally have five arms but some species have more than this – up to 13 or 14. The arms are broad at the base and are not normally abruptly separated from the disc. There are tube-feet often with sucker-like ends along the ambulacral grooves on the underside of the arms radiating from the central mouth (Picton, 1993).

Movement of the sea stars is hydraulic produced by the water vascular system. Water comes into the system via the madreporite. Water circulates from the stone canal to

the ring canal and into the radial canals. The radial canals carry water to the ampullae and contractions of these expand the tube feet. The tube feet latch on to surfaces and move in a wave, with one body section attaching to the surfaces as another release (Hickman *et al.,* 2006).

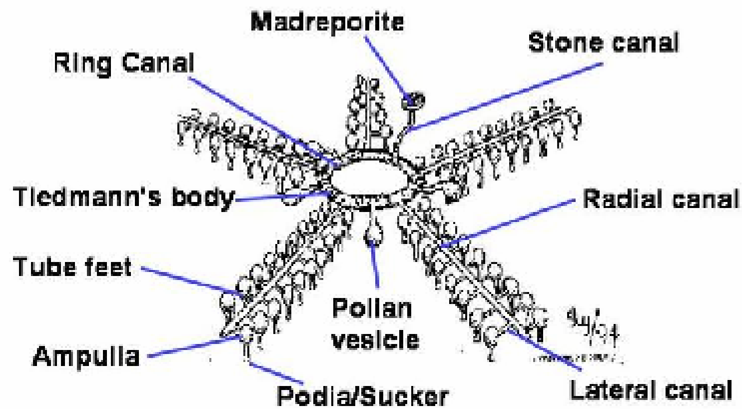


Fig. 5 Water vascular system in starfish (Mulcrone, 2005)

Marthasterias glacialis (Linnaeus, 1758)

Marthasterias glacialis is a large starfish up to 70 cm across, commonly 25 – 30 cm. It has five narrow tapering arms with three rows of longitudinal spines along each arm. The spines are white, usually with purple tips. Spines are surrounded by specialised minute modified spines (pedicellariae), which are used for protection and to gather food. *Marthasterias glacialis* can be dirty brown to pale greyish green in colour with purple tips to the arms. This species may be found in a very wide range of habitats from sheltered muddy sites to fully exposed rockfaces.

Fig. 6 *Marthasterias glacialis*

1.6.2 Holothuroidea (Sea cucumbers)

Sea cucumbers are soft-bodied animals with an elongated body bearing a crown of highly modified tube feet that act as tentacles at one end. In most species the body is covered with tube feet in discrete rows or scattered all over the body (McKenzie, 1988). Exceptions to this are the apodous sea cucumbers which are wormlike animals whose only tube feet are the tentacles. Nearly all holothurians have calcareous spicules embedded in their skin (Picton, 1993).

Several species of aspidichirote sea cucumbers, posses a very specialized defensive system: the so-called Cuvierian tubules (Jourdan, 1883; Mines, 1912; Crozier, 1915).

Cuvierian tubules consist of several hundred tubules whose proximal ends attach to the basal part of the left respiratory tree and whose distal, blind ends float freely in the coelomic cavity. When a sea cucumber is irritated, the animal curves its aboral end toward the irritating object and undergoes a general contraction. The anus opens, the wall of the cloaca tears and free ends of Cuvierian tubules together with coelomic fluid, are expelled through the tear and the anus. The emitted tubules lengthen, instantly become sticky and rapidly immobilize most organisms with which they come into contact (VandenSpiegel and Jangoux, 1987).

***Holothuria forskali* (Della chiaje, 1841)**

Holothuria forskali is a common large sea cucumber. It is usually black in colour but sometimes with yellow mottling especially on the underside. The dorsal papillae may be white-tipped. The animal will readily eject white threads, the cuvierian organs, from the rear end if molested, hence the common name “Cotton-Spinner”. The spicules are very small and rare in the body wall.

Fig. 7 Sea cucumbers *Holothuria forskali*

2. Aim of study

The aim of the study was isolation and characterisation of adhesive proteins from two species of echinoderms: the starfish *Marthasterias glacialis* and the sea cucumber *Holothuria forskali.*

The research was founded by a post-graduate scholarship as part of the "Biocapital" Marie Curie Training Network and undertaken in two laboratories: Integrin Advanced Biosystems (Oban, Scotland) and Johannes Gutenberg University (Mainz, Germany).
The starting point of this research was identification of molecules responsible for adhesion. There has been no previous work that has resulted in anything other than broad classification of the types of molecules involved in adhesion. Isolation and identification of the adhesive protein may make these molecules useful for technological application.
Tests of the adhesive protein under various conditions showed applications of these molecules depend of the environments. Construction of the simply equipment for the adhesive measurement of the liquid were new and quick solution for measurement adhesion properties of the various extracts.

Isolations of the adhesive protein will enable the next target to be addressed which involves finding encoding genes and to produce recombinant adhesive protein which can be commercialised.

3. Materials

3.1 Chemicals

The chemicals and equipment which were used at Integrin Advanced Biosystems in Oban, Scotland and the Institut für Physiologische Chemie and Patobiochemie Johannes Gutenberg-Universität, Mainz, Germany are listed in Table 1.

Acetic acid	Roth, Karlsruhe or Fisher Scientific, Loughborough
Acetone	Roth,Karlsruhe or Fisher Scientific, Loughborough
Acrylamide	Roth, Karlsruhe or Sigma, Irvine
Acid fuchsine	Sigma, Irvine
Alcian blue	Sigma, Irvine
Albumin Fraction V	Roth, Karlsruhe or Sigma, Irvine
Ammonium persulfate	Sigma, Taufkirchen or Fisher Scientific, Loughborough
Aniline blue	Sigma, Taufkirchen
Blocking reagent	Roche, Mannheim
Bradford reagent (Roti Blue Quant)	Roth, Karlsruhe or Sigma, Irvine
5-bromo-4-chloro-3-indylophosphate P-toluidine salt	Roth, Karlsruhe
Bromophenol blue	Serva, Heidelberg or Sigma, Irvine
Chloroform	Roth, Karlsruhe
Dimethylformamide	Applichem GmbH, Darmstadt
Disodium hydrogen phosphate dihydrate	Merck, Darmstadt
Disodium tetraborate decahydrate	Sigma, Taufkirchem

DPX	Sigma, Taufkirchem or Sigma, Irvine
Dithiothreitol (DTT)	Sigma, Taufkirchem or Sigma, Irvine
Eosin Y	Sigma, Taufkirchem or Sigma, Irvine
Ethanol	Roth, Karlsruhe
Ethylenediaminetetra acetic acid (EDTA)	Roth, Karlsruhe or Fisher Scientific, Loughborough
Fluorescent mounting medium	Dako,Hamburg
Formaldehyde	Roth, Karlsruhe or Sigma, Irvine
GeCode Blue Stain Reagent	Pierce, Bonn or Pierce, Blackburn
Glycerol	Sigma, Taufkirchem or Sigma, Irvine
Guanidine Hydrochloride	Merck, Darmstadt or Sigma, Irvine
Glycine	Roth, Karlsruhe
Haematoxylin	Roth, Karlsruhe or Sigma, Irvine
Histoclear	Sigma, Irvine
Hydrochloric acid	Roth, Karlsruhe or Sigma, Irvine
Imperial Stain	Pierce, Bonn or Pierce, Blackburn
Isopropanol	Roth, Karlsruhe or Sigma, Irvine
Magnesium chloride	Sigma, Irvine
Methanol	Roth, Karlsruhe
β-Mercaptoethanol	Sigma, Taufkirchem
P-Nitrotetrazoliurblue chloride	Roth, Karlsruhe
Periodic acid	Sigma, Irvine
Phosphotungstic acid	Sigma, Irvine
Ponceau	Sigma, Irvine
Potassium chloride	Merck, Darmstadt
Propylene glycol	Sigma, Irvine

Roticlear	Roth, Karlsruhe
Rotiphorese	Roth, Karlsruhe
Saccharose	Roth, Karlsruhe
Sephadex	Pharmacia Fine Chemicals, Uppsala
Schiff's Reagent	Sigma, Irvine
Sodium bicarbonate	Roth, Karlsruhe
Sodium chloride	Roth, Karlsruhe or Fisher Scientific, Lougborough
Sodium dodecylsulfate (SDS)	Roth, Karlsruhe or Fisher Scientific, Lougborough
Sypro ruby stain	Invitrogen, Paisley
Technovit 8100	Roth, Karlsruhe
Tetramethylethylenediamine (TEMED)	Roth, Karlsruhe or Sigma, Irvine
(Tris(hydroxymethyl)aminomethane), Tris or (Tris(hydroxymethyl) aminomethane Hydrochloride), Tris - HCl	Roth, Karlsruhe or Sigma, Irvine
Tween 20 (Poly(oxyethylen)$_n$-sorbitan- monolaurate)	Sigma, Taufkirchem
Urea (Diaminomethanal)	Roth, Karlsruhe or Sigma, Irvine

3.2 Equipment

AFM Bioscope I Dimension Head (single piezo) Nanoscope 3a controller Software version 4.43 r 8 2000	Veeco, Santa Barbara, US
Centrifuges: Eppendorf centrifuge 5402 Heraeus Biofuge fresco Sorvall RC 5B	 Eppendorf Hamburg Kendro, Hannau or DJB Labcare Ltd Newport, Pagnell DuPont, Bad Nauheim
Centrifugal Filter Devices Microcon	Milliore, Schwalbach or Millipore Stonehause
Centrifugal concentrator Amicon	Millipore, Schwalbach
Cuvets	Roth, Karlsruhe
Eppendorfs (2 ml, 1 ml, 0.5 ml)	Roth, Karlsruhe or Fisher Scientific Lougborough
Electro-eluter model 422	Bio-Rad, Munchen
Electrophoresis apparatuses	Bio-Rad, Munchen
Glass equipment	Shott, Mainz or Fisher Scientific, Lougborough
Glass beads	ElringKlinger Kunststofftechnik GmbH, Grossostheim-Ringheim
Heatblock Thermostat 53 20	Eppendorf, Hamburg
Heatplate	IKA Labortechnik, Steufen
Homogenizer Yellow Line DJ 25 Basic	Yellow Line, Northamptom
HPLC 717 plus Autosampler 996 Diode Array 600 controller Software Empower	Waters, Manchester

Columns: Biosep- SEC S 4000 Synergi Max RP C12 Reverse Column Biobasic 18, 4µl	 Phenomenex, Hurdsfileld Ind.Est Thermoelectron, Runcon
Incubator model 400	Memment, Schwalbach
Light microscope Olympus AHBT3	Olympus, Hamburg
Pipettes (2, 10, 20, 100, 200, 1000 µl)	Gilson, France
pH-meter Type CG 840	Schott, Mainz
Slide-A-Lyzer Mini Dialysis Cassette 0.5-3 ml	Pierce, Blackburn
Spectrophotometer SmartSpec Plus	Bio-Rad, Munchen
Spectrophotometer Titertek Multiskan Plus	Bartolomey Labortechnik, Rheinbach
Stirrer	Heidolph, Kehlheim
Shaker Polymax 1040	Heidolph, Kehlheim
Trans-Blot SD Semi-Dry Electrophoretic	Bio-Rad, Munchen
Tecnai 12 microscope	FEI Electron Optics, Eindhoven; Netherlands
Vortexer	Labotech, Wiesbaden
Whatman filter	Whatman GmBH, Dassel
Whatman paper	Whatman GmBH, Dassel
Wyko NT1100 Optical Profiling System Software Veeco Vision	Veeco, Cambridge

3.3 Antibodies

Polyclonal Antibody no N374 (against silk fibroin from *Mytulis edulis)*	Lab, Mainz
Polyclonal Antibody no N384 (against adhesive protein from *Holothuria forskali)*	Lab, Mainz
Cy3-conjugated goat anti-*rabbit*	Dianova, Hamburg
Anti-rabbit IgG (whole molecule) alkaline phosphatase	Sigma-Aldrich, Taufkirchem

3.4 Markers

PeqGOLD Protein Marker I	PeqLab, Erlangen
Precision Plus Protein Standards, Dual Color	Bio-Rad, München
TriChromRanger™ Prestained Molecular Weight Marker Mix	Pierce, Blackburn

3.5 Kits

GelCode® Glycoprotein Staining Kit	Pierce, Blackburn
PAGE prep® Advance Kit	Pierce, Blackburn
ReadyPrep™ 2-D Cleanup Kit	Bio-Rad, Munchen
Silver stain kit	Pierce, Blackborn
Immunoprecipitation Kit	Pierce, Bonn
2-D Quant Kit	Amersham Biosciences Europe GmbH, Freiburg

3.6 Experimental animals

3.6.1. Starfish (*Marthasterias glacialis*, Asteroidea, Echinodermata)

Marthasterias glacialis was collected by divers from sea lochs on the West Coast of Scotland (Loch Fyne). Animals were maintained in a re-circulating aquarium at 8-10 °C in natural filtered sea water and fed with mussels (*Mytulis edulis).* These starfish were not kept longer than 4 weeks, because after this time they stopped producing mucus.

3.6.2 Sea cucumber (*Holothuria forskali,* Holothuroidea, Echinodermata)

Holothuria forskali were caught Adriatic Sea at the coast of Istria near Rovinj (Croatia) with a dragnet at a depth 20m in summer (August 2007) (See Fig. 8). Cuvierian tubules were removed from the sea cucumber and the animals released back into the natural environment.

Fig. 8 Collection of sea cucumber using dragnet

4. Methods

4.1. Methods use for isolation adhesive material

4.1.1 Collections of adhesive material

4.1.1.1 Starfish

Removal of starfish tube feet: after anaesthetising the starfish with 2% $MgCl_2$, the suckers from tube feet were removed and stored frozen, prior to extraction. This provided a whole tissue sample of the target cells.

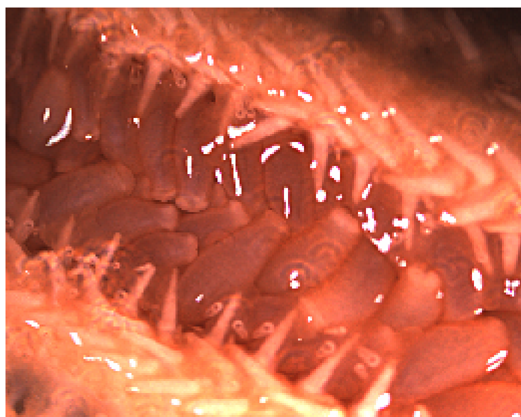


Fig. 9 Tube feet of starfish

Collection of the footprint on glass: starfish adhered to large glass plates and slides and the resulting footprint was collected by scalpel or razor blade into sterile tubes. This material was stored frozen prior to extraction.

Collection of the footprint from glass using a wooden stick: to obtain pure footprint and eliminate any contaminating material the footprint was collected separately from the glass plates using a cocktail stick.

4.1.1.2 Sea cucumber

Cuvierian tubules was removed from specimens previously anaesthetized for 1 h in saturated solution of urethane and kept in methanol and transport in 4 °C to laboratory in Mainz.

Fig. 10 Dissection of sea cucumber

4.1.2 Extraction of adhesive material

4.1.2.1 Starfish

Four buffers were used to solubilise the tissue and footprint material:

0.063 M Tris-HCl, 2% SDS 1.6 M urea, 0.1 M DTT, pH 8	6 M guanidine-HCl, 0.5 M DTT, 0.05 M Tris, pH 8.8	6 M urea, 0.05 M Tris-HCl, pH 8	2 M urea, 0.063 M Tris-HCl, 0.1 M DTT, pH 8

Cut tube feet (1 g) were mixed with buffer (3ml) and homogenized.

After homogenization, the suspension was centrifuged for 15 min (5000 x g). Supernatant was collected. Various dialysis buffers were tested to clean-up the extract prior to gel electrophoresis:

0.05 M Tris-HCl, pH 8	0.063 M Tris-HCl, 5% EDTA pH 8	PBS

A dialysis cassette was used for dialysis (0.5 ml, Pierce). Homogenised extract was injected into the cassette and put in 1 l of dialysis buffer and stirred overnight. After this time the extract were concentrated using spin column. Cut off spin columns (Millipore) were used as an initial step to clean up and partition the extract.

4.1.2.2. Sea cucumber

Collected organs were dried by lyopholization. Dried organs were ground in a mortar containing liquid nitrogen. Several buffers were used in an attempt to solubilise the tissue.

4 M urea buffer, 0.5 M Tris, pH 7.5	0.5 M NaCl, 5 mM Tris, 7 mM Na_2SO_4, 0.4 mM $NaHCO_3$, 20 mM EDTA	150 mM NaCl, 1.5% NP_{40}, 0.1% SDS, 0.1% DOC, 50 mM Tris, pH 8.	Sample buffer (100 mM Tris, pH 6.8, 2% SDS, 5% ß-mercaptoethanol, 15% glycerol, 0.006% Bromophenol blue)

2 g of dried material was stirred with 50 ml of buffer overnight at 4 ºC. The homogenized suspension was then centrifuged at 14 000 x g for 15 min. The supernatant was collected and filtered with a 2 µm filter (Millipore). Supernatant was dialysed overnight using dialysis tube in 5 l of water, changed every 3 hours. A concentration tube (50 ml) (Millipore) was used to concentrate the dialysed material then it was, centrifuged to obtain 1ml of extract (speed 4000 x g).

4.1.3 Purification of crude extract for SDS-PAGE analysis

4.1.3.1 PAGE prep® Advance Kit

The PAGE prep® Advance Kit was used to concentrate samples for SDS-PAGE analysis while removing interfering chemicals.

Procedure:

The resin in the PAGE prep® Advance Kit binds protein in the presence of dimethylsulfoxide (DMSO). 20 µl of resin slurry was mixed with 300 µl of sample and 300 µl of 100% DMSO. The mixture was incubated for 3-5 min at room temperature with occasional mixing to ensure maximum protein adsorption to the resin. To remove unbound residues, samples were centrifuged (2,000 x g for 2 min). The precipitated mixture was washed in diluted DMSO (50%). The wash solution was prepared by mixing 6 ml of DMSO with 6 ml of water and 300 µl added to the resin. To obtain a homogeneous suspension the mixture was vortexed. To increase purity of the sample, the centrifugation step (2,000 x g for 2 min) and washing steps were repeated three times.

After several washings 50 µl of Elution Buffer was added to the resin and sample. This was then incubated at 60 °C for 5 min to solubilise the protein. After solubilisation of the protein any impurities stayed in solution. Centrifugation was used to separate pure sample from any residues (2,000 x g for 2 min). The sample was then combined with 5 x sample buffer. For denaturation, the sample was heated at 95 °C for 5 min and then the SDS-PAGE undertaken. The eluted proteins were then combined with sample buffer and analyzed on the SDS-PAGE system of choice.

4.1.3.2 Ethanol precipitation

Ethanol precipitation is a useful method to concentrate proteins and remove guanidine hydrochloride before SDS-PAGE. This method was also used to concentrate DNA. DNA and proteins are polar and soluble in water because water is polar but they are insoluble in the relatively nonpolar ethanol.

Procedure:

Proteins were initially precipitated by adding a large volume of 100% ethanol (100 µl of protein solution added to 900 µl of cold ethanol 100%). Cooling on ice helped precipitate protein (at least 10 min at –20 ºC). To remove any impurities, the mixture was spun (15 min 4 ºC at maximum speed (15000 x g). In the next step, 70% – 80% ethanol was added to the precipitated pellet and gently mixed to re-suspend the pellet. This allowed the 20% water to access the salts present in the pellet. The suspension was centrifuged and supernatant was removed. Samples were dried under vacuum (speed vac) or dry air to eliminate any ethanol residue (this was tested by simply smelling the tubes until no ethanol could be detected). Samples were re-suspended in a minimal volume (20 µl) of sample buffer. Isopropanol was sometimes used instead of ethanol as the precipitation efficiency of the isopropanol is higher. However, isopropanol is less volatile than ethanol and needed more time to air-dry in the final step.

4.1.3.2 TCA/Acetone precipitation

This precipitation was used to clean and/or concentrate a protein sample or to enable transfer of a protein sample from one buffer to another. The pellet obtained after precipitation was purified with acetone, which removed residues of impurity.

Procedure:

To obtain the best results, a fresh vial of 100% trichloroacetic acid (TCA) was prepared for each sample. 100% TCA was added to each sample to reach a final TCA concentration of 10%. The sample turned milky white if proteins were present. To get maximum precipitation, the sample was incubated on ice for 1 hour. Centrifugation was used to separate the sample(s) from any impurity, leaving a white pellet at the bottom of the tube. To wash the protein pellet, prechilled –20 °C acetone (800 µl) was used. The proteins did not dissolve in the acetone, they were only suspended. This was necessary to ensure removal of the TCA. The sample was incubated overnight at –20 °C in acetone. After this incubation, the samples were centrifuged at 6500 x g in 4 °C for 10 min. The pellet was then washed in a new portion of acetone three times. After the final

centrifugation, the acetone was discarded and the pellet completely dried to remove any residues of acetone. The pellet was then dissolved in the sample buffer.

4.1.3.3 ReadyPrep™ 2-D Cleanup Kit

The ReadyPrep™ 2-D cleanup kit was used to facilitate the preparation of low conductivity samples suitable for isoelectric focusing (IEF) and 1 or 2-D gel electrophoresis. This kit concentrates proteins from samples that are too dilute, allowing for higher protein loads and improved spot or band detection.

Procedure:

100 µg of the protein was mixed with 300 µl precipitating agent 1 and incubated on ice for 15 min to allow the protein to precipitate. The obtained mixture of protein was added to 300 µl precipitating agent 2. After mixing and vortexing, the sample was centrifuged at maximum speed (12,000 x g) for 5 min to form a tight pellet. After discarding the supernatant, 40 µl of wash reagent 1 was added to the sample pellet. This was washed with wash reagent 1 then distilled water. After this, 1ml of washing reagent 2 (prechilled at -20 °C for at least 1 h) and 5 µl of wash second additive was incubated with the sample for 30 min in –20 °C with mixing and vortexing every 10 min. Next the tube was centrifuged at top speed for 5 min to form a tight pellet. The obtained pellet was pure protein. The pellet was air-dried at room temperature for no more than 5 min and then re-suspended by adding an appropriate volume of sample buffer.

4.1.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE has a number of uses, which include the establishment of protein size, protein identification, determination of sample purity, identification of disulfide bonds, quantification of proteins and blotting application.

Acrylamide gels are formed by polymerising acrylamide with a cross-linking agent (bis-acrylamide) in the presence of a catalyst/chain initiator mixture thus producing a cross-linked matrix with particular pore size. The size of the pores of the matrix is determined by the concentration of acrylamide and N, N'-methylenbisacrylamide so that

the range of separations can be selected as required. TEMED (N, N, N, N'-tetramethylenodiamine) is most commonly used as the catalyst, and persulphate ion ($S_2O_8^{2-}$) as the initiator. SDS is the most common dissociating agent used to denature native proteins to individual polypeptides. All proteins migrate to the anode. B-mercaptoethanol is present in the loading dye to destroy disulfide bonds, thus breaking the only known covalent bond between the polypeptide chains. The migration distance during the electrophoresis is then linearly dependent on the logarithm of the molecular size (Weber and Osborn, 1969). Through using protein of known molecular mass, it was possible to elevate the molecule mass of the polypeptide chains.

In a discontinuous system, such as used in this study, a non-restrictive, large pore gel (stacking gel) is layered on top of a separating gel (resolving gel). Each gel is made with a different buffer and the tank buffers are different from the gel buffers. For performing PAGE, vertical electrophoresis tanks from Mini Protean II from Bio-Rad were used (minigels: 7 cm x 8 cm x 1 mm). The gels were used directly or stored under humid conditions at 4 °C until use.

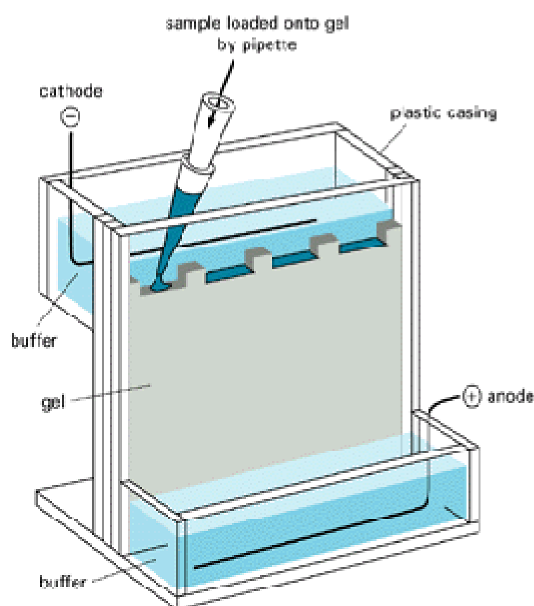


Fig. 11 System used for SDS-PAGE

Procedure

Separation of the samples was done in 12-15% resolving gels, overlaid with an 8-10% stacking gel (Table 2).

Table 2 Pipetting scheme for 2 minigels.

	12% resolving gel	8% stacking gel	15% resolving gel	10% stacking gel
40% acrylamide /bis stock	3 ml	1 ml	3.75 ml	1.25 ml
1.5 M tris HCl (pH 8.8)	2.5 ml		2.5 ml	
1 M tris HCl (pH 6.8)		1.25 ml		1.25 ml
10% SDS	0.1 ml	0.05 ml	0.1 ml	0.05 ml
glycerol	0.2 ml		0.2 ml	
H_20 distilled	4.1 ml	2.55 ml	3.35 ml	2.3 ml
APS 10% (w/v)	0.1 ml	0.05 ml	0.1 ml	0.05 ml
TEMED	0.01 ml	0.005 ml	0.01 ml	0.005 ml

4.1.4.1 Semi-native PAGE

While the separation of proteins during an SDS-PAGE exclusively occurs due to their molecular size, during a native PAGE, untreated proteins are separated electrophoretically due to their molecular size combined with their net charge and conformation (tertiary structure). In Table 3 solutions used in different SDS systems are listed. Note that the buffers, used for native/semi-native PAGE, contained neither or less SDS and β- mercaptoethanol and that the sample denaturation step at 95 °C was omitted.

Table 3 Solutions used depending on the system: System specific solutions for SDS, semi-native PAGE

	SDS-PAGE	Seminative PAGE
Resolving buffer	1.5 M Tris/HCl pH 8.8 0.1% SDS	1.5 M Tris/HCl pH 8.8 0.1% SDS
Stacking gel buffer	1.0 M Tris/HCl 0.1% SDS pH 6.8	1.0 M Tris/HCl 0.01% SDS pH 6.8
Running buffer	25 mM Tris, pH 8.3 192 mM glycine 20% MeOH 0.1% SDS	25 mM Tris, pH 8.3 192 mM glycine 20% MeOH 0.1% SDS
Loading dye (4 x)	0.5 M Tris pH 6.8 40% glycerol 8% SDS 20% _-mercaptoethanol Bromophenol blue	0.5 M Tris pH 6.8 40% glycerol - - Bromophenol blue

4.1.5 Stains for SDS PAGE

4.1.5.1 GelCode® Blue stain reagent

GelCode® Blue Stain Reagent (Pierce) utilizes the colloidal properties of Coomassie® G-250 for protein staining on polyacrylamide gels. The mechanism of Coomassie dye binding to proteins is not completely understood, however, it depends in part on basic and hydrophobic residues. For this reason, binding varies widely among proteins.

Procedure:

After electrophoresis, the gel was washed twice with distilled water with gentle shaking for 15 min to remove any SDS residues. For an 8 x 10 cm mini gel, 20 ml of GelCode® Blue Stain Reagent was used. Stain intensity reaches a maximum within approximately 1 h. After staining, gels were washed with distilled water (1 – 2 h) for optimal results. This step enhances stain sensitivity as weak protein bands continue to develop.

4.1.5.2 Glycoprotein stain

Glycoproteins consist of carbohydrate moieties covalently linked to a polypeptide backbone. These diverse proteins may have a carbohydrate component that represents < 1% to > 80% of the total weight. Sugars that commonly occur in glycoproteins include galactose, mannose, glucose, N-acetylglucosamine, N-acetylgalactosamine, sialic acid, fucose and xylose.

The GelCode® Glycoprotein Staining Kit detects glycoprotein sugar moieties in polyacrylamide gels and on nitrocellulose membranes. When treated with Oxidizing Reagent (periodic acid), glycols present in glycoproteins are oxidized to aldehydes.

After completing the procedure, the glycols are stained, yielding magenta bands with a light pink or colourless background.

Procedure:

After electrophoresis, the gel was fixed by completely immersing it in 100 ml of 50% methanol for 30 min. The gel was washed twice by gently agitating with 100 ml of 3% acetic acid for 10 min. Next step was transfer to 25 ml of Oxidizing Solution and the gel was then gently agitated for 15 min followed by gentle agitation with 100 ml of 3% acetic acid for 5 min. The gel was then transferred to 25 ml of GelCode® Glycoprotein Staining Reagent and gently agitated for 15 min. After this time, the gel was transferred to 25 ml of Reducing Solution and gently agitated for 5 min. The gel was washed extensively with 3% acetic acid and then with ultrapure water. Glycoproteins appear as magenta bands.

4.1.5.3 Silver stain

SilverSNAP® Stain Kit II is a rapid and sensitive silver stain system for protein detection in polyacrylamide gels. Most proteins are easily detectable at low nanogram or subnanogram amounts, and background staining levels are very low. The mechanisms underlying silver staining of proteins in gels are relatively well understood.

Basically, protein detection depends on the binding of silver ions to the amino acid side chains, primary the sulfhydril and carboxyl groups of proteins (Switzer *et al.*, 1979; Oakley *et al.,* 1980; Merril *et al.*, 1981; Merril *et al.*, 1986), followed by reduction to free metallic silver (Rabilloud, 1990; Rabilloud, 1999).

Procedure:

After washing gel with ultrapure water, the gel was fixed 2 x 15 min in 30% ethanol: 10% acetic acid solution. After fixing, the gel was washed 2 x 5 min in 10% ethanol, then 2 × 5 min in ultrapure water. The gel was then sensitized (Sensitizer Working Solution (50 l Sensitizer with 25 ml water) for 1 min and then washed 2 x 1 min with water. The gel was stained for 30 min (Stain Working Solution (0.5 ml Enhancer with 25 ml Stain)).

After staining, the gel was washed 2 x 20 sec with ultrapure water, and the stain then developed (Developer Working Solution (0.5 ml Enhancer with 25 ml Developer)) gel for 2 – 3 min until bands appear. The reaction was stopped with 5% acetic acid for 10 min.

4.1.5.4 Imperial™ Stain

Imperial™ Protein Stain is a Coomassie R-250 dye-based reagent for protein staining in polyacrylamide gels. This sensitive (≤ 3 ng) stain produces an intense colour that photographs well. This reagent stains only protein and allows bands to be viewed directly in the gel during the staining process.

Procedure:

Gel were washed three times per 5 min in ultra pure water to removed any SDS residues and put in Imperial Stain (20 ml for 8 x 10cm gels) to stain. After 30 min – 2h (depends on amount loaded on gel) bands developed. To achieve the desired level of sensitivity,

the gel must be stained for specific time (10 min – 2 h). To de-stain ultrapure water was used (15 min – overnight).

4.1.5.5 Sypro Ruby Protein Gel Stain (Invitrogen)

SYPRO Ruby dye is a permanent stain composed of ruthenium as part of an organic complex that interacts noncovalently with proteins. SYPRO Ruby Protein Gel Stain provides a sensitive, fluorescence-based method for detecting proteins in one-dimensional and two-dimensional sodium dodecyl sulfate-polyacrylamide gels.

Procedure:

Gels were fixed with 50% methanol then rinsed with 7% acetic acid buffer twice for 30 min. After this time, the gel was stained in Sypro Ruby solution (60 ml) overnight. Next step was washing the gel for 30 min in 10% methanol, 7% acetic acid. Proteins were visualized using a 300 nm UV transilluminator.

4.1.6 Antibody production

Polyclonal antibodies (PoAb) were raised against adhesive proteins by immunization in female rabbits (White New Zealand) as described (Schutze *et al.*, 2001). PoAb are derived from different B-cell lines. They are a mixture of immunoglobulin molecules secreted against a specific antigen, each recognizing a different epitope. Injection of antigen (900 µg/ml) into the rabbit induces the B-lymphocytes to produce IgG immunoglobulins specific for the antigen. After three boosts the serum is collected and screened in a conventional ELISA assay as well as by Western blotting.

4.1.7 Western blot

A Western blot (alternatively known as an immunoblot) is a method to detect a specific protein in a given sample of tissue homogenate or extract.

The proteins move from within the gel onto the membrane (PVDF or nitrocellulose) while maintaining the organization they had within the gel. As a result of this "blotting"

process, the proteins are exposed on a thin surface layer for detection. The membrane has non-specific protein binding properties (i.e. it binds all proteins equally well). Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein.

Procedure:

The samples and pre-stain molecular marker were loaded on the 12% or 15% SDS PAGE. Separated proteins were transferred to PVDF membrane (Immobilon) by semi-dry blotting, using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Gel (Bio-Rad) at 60 mA in transfer buffer for 60 min. The membrane (Millipore) was activated in methanol and wetted in blot buffer before transfer. The membrane was stained with Ponceau and de-stained in water. Non-specific binding sites were blocked by 1% (v/v) Blocking solution in TBST for 1 h at room temperature or at 4 °C overnight. After washing (2 x 5 min) with TBST the blots were incubated with primary antibody (against silk fibroin 1:1000 or against adhesive protein from Cuvierian tubules 1:1000) with shaking for 1 – 1.5 h at the room temperature. Unbound reagents were removed and background was reduced by three washing steps, 5 min each, in TBST with shaking. Subsequently, anti-rabbit IgG-alkaline phosphatase conjugate (dilution 1:10000 in blocking buffer) was added to detect bound proteins of interest. After 1 h of incubation at room temperature, the membranes were repeatedly washed three times with TBST and three times with TBS. After equilibration in P_3 for 10 min the antibody-protein complexes are visualized with colorimetric substrates NBT/BCIP.

Blotting (transfer) buffer	25 mM Tris 192 mM glycine 20% methanol (v/v)
Blocking solution	1% Blocking-Reagent in TBS (v/v)
TBS	10 mM Tris, pH 8.0 150 mM NaCl
TBST	0.1% Tween 20 in TBS (v/v)
P_3	100 mM Tris, pH 9.5 100 mM NaCl

Antibody solution	0.5% blocking solution in TBST (v/v)

4.1.8 Neutralisation of antibody

Antibodies were neutralized and made more by adding solutions of the antibodies to extract containing the antigen. Antibody bound to the antigen in solution. When the membrane was incubated with neutralized antibody, only very specific antibodies which are in excess in the solution bound to the protein. This resulted in very specific antibodies being obtained.

Procedure:

The neutralised antibody was prepared as follows:
2.5 µl of Blocking solution (Roche)
2.5 µl of TBS
5 ml of extract of Cuvierian tubules diluted 1:5
10 µl of antibody no. N 384 against adhesive protein from Cuvierian tubules
Mixture was incubated for 2.5 hours in room temperature.
The Western blot was prepared as in procedure 4.1.7. After blocking, the membrane was incubated with the neutralised mixture for 1 h and standard protocol was then followed.

4.1.9 Immunoprecipitation

The Seize® X Immunoprecipitation Kit offers an improvement over the classical method of immunoprecipitation (IP) by immobilizing the antibody to the Protein A gel using the cross-linker DSS. This procedure results in a permanent affinity support with a properly oriented antibody. The crude sample is then incubated with the immobilized antibody to form the immune complex. The affinity support is washed by centrifugation using a Handee™ Spin Cup Column and the remaining antigen is dissociated from the antibody using an elution buffer. The primary antibody does not contaminate the final antigen preparation and the immobilized antibody support is preserved for future IPs.

Procedure:

Immobilized Protein A and reagents were equilibrated to room temperature. To the dry-blend buffer added 500 ml of ultrapure water. 0.4 ml of the Immobilized Protein A (50% slurry) was added into one of the Handee™ Spin Cup Columns and placed inside a Handee™ Microcentrifuge. Tube and the sample centrifuged (5 min, 5000 x g). In this step, protein A bound to the agarose gel. To remove unbound protein A, the gel was washed by adding 0.4 ml of Binding/Wash Buffer to the spin cup containing the gel.

After several washings, 50 µl of purified antibody against adhesive protein from Cuvierian tubules (Mainz) prepared in 0.4 ml of Binding/Wash Buffer was applied. The tube was placed on a rocker for at least 15 min to allow the antibody to bind to the gel and the sample then centrifuged. To remove any unbound antibody, the gel was washed several times with Wash/Binding buffer. To the tube of No-Weigh™ DSS 80 µl of DMSO was added. 25 µl of the DSS solution was added to the spin cup containing the bound antibody support. Samples were then gently mixed for 60 min. The tube was centrifuged (3 min, 5000 x g). 500 µl of ImmunoPure® Elution Buffer was added to the spin cup. The tube was then centrifuged (1min 5000 x g). The washing step was repeated four additional times to quench the reaction and to remove excess DSS and any uncoupled antibody. The sample was diluted 1:1 with Binding/Wash Buffer and 200 µl of the sample to be purified was added to the spin cup. The incubated sample was gently mixed for at least 1 h at room temperature or overnight in 4 ºC.

After discarding the extract, the agarose gel was washed several times to remove any contamination. For elution, added 190 µl of ImmunoPure® Elution Buffer was added to the spin cup and centrifuged (3 min 5000 x g). Sample was eluted within the first three fractions. The first three fractions were assessed by SDS-PAGE. 20 µl of the sample was mixed with Lane Marker Sample Buffer (5 µl) and heated to denaturate the sample. The sample was then ready to be applied onto the electrophoresis gel.

4.1.10 Electro-elution

The Model 422 Electro-Eluter solves many of the problems of conventional elution of protein from gel slices by providing easy assembly and eliminating sample loss due to leakage. The Electro-Eluter consistently yields between 70% and 100% of the starting material, depending on the type of elution. The samples are collected in 400-600 µl volumes. The small volume of eluted material makes subsequent manipulations easy.

Procedure:

The membrane caps were soaked at 60 °C in the elution buffer for at least 1 hour prior to use. Four of glass tubes were taken and one frit placed in the bottom of each tube. One pre-wetted membrane cap was placed in the bottom of each silicone adaptor. The adaptor was filled with elution buffer. The silicone adaptor with the membrane cap was slide onto the bottom of the glass tube with frit. See Fig. 12

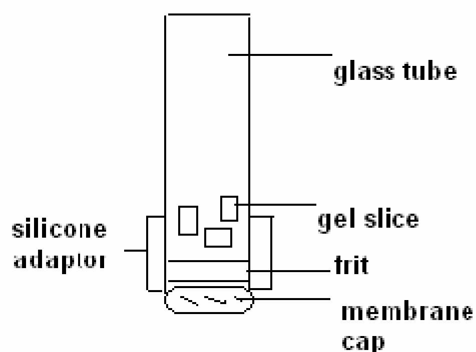


Fig. 12 Side view of Model 422 Electro-Eluter assembly.

The vertical glass tube was filled with elution buffer. The negative electrode was at the top. The positive electrode was below the membrane cap. Macromolecules were carried by the electrical current out of the gel slice, through the frit and into the membrane cap. The molecules were retained by a dialysis membrane which is moulded into the cap.

Each tube was filled with elution buffer and the gel slice placed in the tube. To increase sample recovery, several bands were excised from the gel and minced, while

ensuring that the height of the gel within the glass tube never exceeded approximately one cm.

The entire module was placed into the buffer chamber. Elution was done at 8-10 mA/glass tube. The eluted protein was concentrated with a cut off column (Millipore) to 50 µl. SDS-PAGE and Western blot were carried out to check purity of the eluted protein.

4.1.11 Glass beads

Glass beads were used for isolation of adhesive protein from crude extract. Adhesive protein should stick to glass beads during incubation with shaking and vortexing. Non-denturating buffer did not remove this adhering protein from the surface of the glass during washing steps. Proteins which do not possess adhesive force should be washed off from the glass beads surface by the buffer leaving only bound protein remaining after the washing step which was then recovered using a protein denaturating buffer solution which breaks the bonds between the glass beads and the adhesive protein.

Various concentrations of extract

Adhesion depends on various conditions. In this experiment the impact of various concentrations of the extract buffer for adhesion to glass beads was studied.

Procedure:

2 g of dried Cuvierian tubules was extracted in 4 M urea in 4 ºC with stirring. The resulting suspension was centrifuged at 5000 x g. The supernatant was then collected.
The extract obtained was diluted to three different dilutions:
3 M urea extract (500 µl of 4M urea extract and 166 µl of Tris 0.05 M)
2 M urea extract (500 µl of 4M urea extract and 500 µl of Tris 0.05 M)
1 M urea extract (500 µl of 4M urea extract and 1500 µl of Tris 0.05 M)

Glass beads (2 mm diameter size) were washed with 4 M urea buffer and 1 g of glass beads was added to each of four tubes containing the same amount of protein but various concentrations of urea (4 M, 3 M, 2 M , 1 M urea). Tubes were shaken for 2 hours and vortexed every 10 min to allow the protein to adhere to the beads. After this

time, the extract was discarded from the tubes and the glass beads were washed with 0.05 M Tris (1 ml) to remove all unbound proteins and remaining extract. After removing Tris buffer, 50 µl of Sample Buffer 4 x (100 mM Tris, pH 6.8, 2% SDS, 5% ß-mercaptoethanol, 15% glycerol and 0.006% Bromophenol blue) was added to the tubes. The glass beads were then boiled and shaken for 15 min. in 95 ºC in a thermomixer. After this time, the supernatant was collected and 20 µl was loaded on 12% SDS-PAGE. The gel was run and stained with GelCode Blue Stain Reagent and de-stained with water. The gel was scanned using an Odyssey Scanner and, by employing the Odyssey v.1.2 software it was possible to quantify bonds.

Various sizes of glass beads

Correlation between sizes of the beads and adhesion was tested using four different sizes of beads (70-100 µm; 150-300 µm; 300-400 µm and 2 mm).

The glass beads were washed with 4 M urea buffer. To each tube containing 1 g of the particular beads 1 ml of 4 M urea extract was added. The mixture was shaken for 2 hours and vortexed every 10 min to allow protein to adhere to the beads. After this time, the extract was removed and the glass beads were washed three times with 1 ml of 0.05 M Tris buffer to remove the extract and all not bound proteins. Tris buffer was removed and 50 µl of Sample Buffer 4 x (100 mM tris, pH 6.8, 2% SDS, 5% ß-mercaptoethanol, 15% glycerol, 0.006% Bromophenol blue) was added to the tubes. The glass beads were boiled and shaken for 15 min in 95ºC in the thermomixer for denaturation. From each tube 20 µl was taken and loaded on 12% SDS-PAGE gel. After staining, the gel was scanned using Odyssey Scanner and bond quantification was done by Odyssey v.1.2.

4.1.12 Gel filtration

Gel filtration chromatography is used for separating proteins, peptides, and oligonucleotides on the basis of size. Molecules move through the column and small molecules diffusing into porous beads, the larger molecules enter less or not at all and move through column more quickly than the smaller molecules. Gel Filtration Chromatography may be used for several applications like analysis of molecular size, for

separations of components in a mixture or for salt removal or buffer exchange from a preparation of macromolecules.

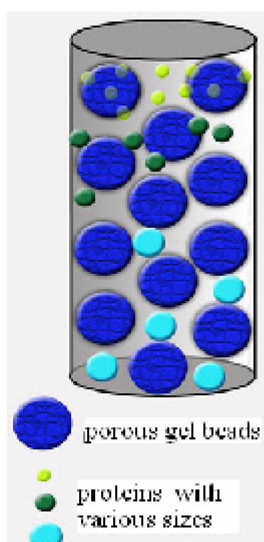


Fig. 13 Scheme of gel filtration

Procedure:

Sephadex G-50 was used for preparation of the column. 2 g of the powder was mixed with an excess of distilled water. The mixture was left for several hours to expand the beads, and then the mixture was poured into the column (1 cm x 15 cm). The column was then washed with 4 M urea. A mixture of BSA, silk fibroin and carbonic anhydrase in 4M urea was used for calibration of the column. Proteins were eluted using 4 M urea. Retention time of each sample was recorded. Presence of protein in each fraction was checked by Bradford assay. 1 ml 4 M urea extract of the Cuvierian tubule was loaded on calibrated column of Sephadex G-50. Protein was eluted using 4 M urea. Forty 500 μl fractions were collected. Each fraction was assayed with Bradford Assay to check the concentration of protein. Fractions with a high concentration of proteins were loaded on two 12% SDS PAGE gels. One gel was transferred to the PDVF membrane. Another gel was stained with Gel Code Blue Stain Reagent and de-stained with water and scanned

with Odyssey Scanner. Transferred gel was blocked and then incubated with antibody against the adhesive protein. It was then incubated with secondary antibody and detection was by with the NBT/BCIP (Procedure 4.1.7).

4.1.13 Digestion with trypsin (In-Gel Tryptic Digestion Kit, Pierce)

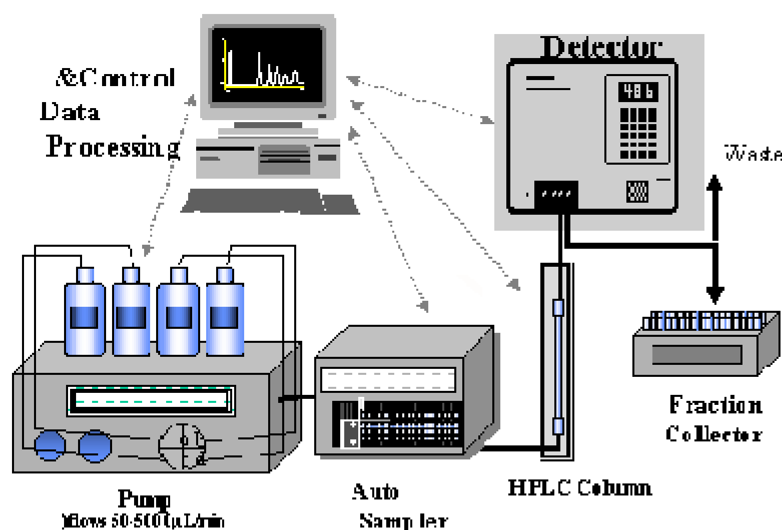
In-gel digestion, coupled with mass spectrometric analysis is a powerful tool for the identification and characterization of proteins. The kit includes modified trypsin, de-staining buffers, digestion buffers, reduction reagents and alkylation reagents. The methodology of this kit has been designed to function with a wide range of protein band concentrations producing complete and accurate digest for dependable mass spectrometric (MS) analysis.

Procedure:
Polyacrylamide gel was prepared by procedure 4.3. Gels were stained with GelCode Blue Stain Reagent (procedure 4.4.1). A scalpel was used to excise the protein band of interest from the 1-D gel. To 2 x 2 mm pieces added 200 µl De-staining Solution. The sample was incubated at 37 ºC for 30 min with shaking to remove the stain. This step was repeated several times. Reducing Buffer was prepared by mixing 3.3 µl of TCEP (tris (2-carboxyethyl) phosphine) with 30 µl of Digestion Buffer for each digest to be performed. The final TCEP concentration was ~50 mM. 30 µl of Reducing Buffer was added to the tube containing the sample and incubated at 60 ºC for 10 min. The samples were allowed to cool and then the Reducing Buffer was removed from the tube and discarded. Alkylation Buffer was prepared by dissolving 7 mg of IAA (iodoacteamide) in 70 µl water to make a 5 x stock (~500 mM final concentration). 7 µl of the 5x stock solution was diluted with 28 µl of Digestion Buffer for each digest being performed. 30 µl of Alkylation Buffer was added to the tube containing the gel pieces. The sample was incubated in the dark at room temperature for 1 h then Alkylation Buffer was removed and discarded from each tube. The sample was washed by adding 200 µl Destaining

Buffer to the tube and then incubated at 37 ºC for 15 min with shaking. The Destaining Buffer was then removed from the tube. The wash steps were repeated and then the gel pieces shrunk by adding 50 µl of acetonitrile and incubating the samples for 15 min at room temperature. The acetonitrile was carefully removed and the gel pieces allowed air-drying for 5-10 minutes. Activated Trypsin was prepared and then 1 µl of the Trypsin Working Solution was diluted with 9 µl of Digestion Buffer for each sample being processed giving a final concentration of ~10 ng/µl. The activated Trypsin was stored on ice until use. 10 µl of Activated Trypsin solution was added to each tube and the sample incubated at room temperature for 15 min. 25 µl Digestion Buffer to the tube was then added to each tube and the samples incubated at 37 ºC for 4 h or at 30 ºC overnight with shaking. The digestion mixture was then removed and placed in a clean tube. The mixture was kept in –20 ºC for future analysis.

4.1.14 HPLC

High-performance liquid chromatography (HPLC) is a form of column chromatography frequently used in biochemistry and analytical chemistry. It is also sometimes referred to as high-pressure liquid chromatography. High performance liquid chromatography is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster. It also allows using a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture. The other major improvement over column chromatography concerns the detection methods which can be used. These



methods are highly automated and extremely sensitive. A scheme of the equipment is show on Fig.14

Normal phase HPLC:

Fig.14 Scheme of the HPLC

Normal phase HPLC (NP – HPLC) was the first of HPLC chemistry developed and separates analytes based on their polarity. This method uses a polar stationary phase and non-polar mobile phase and is used when the anlayte of interest is fairly polar in nature. The polar analyte associates with and is retained by the polar stationary phase. Adsorption strengths increase with an increase in analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time.

Reversed phase chromatography

Reversed phase HPLC (RP – HPLC) consists of a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica which has been treated with Rme_2SiCl where R is a straight chain alkyl group. The retention time is therefore longer for molecules which are more non-polar in nature, allowing polar molecules to elute more readily. Retention Time (RT) is increased by the addition of the polar solvent to the mobile phase and decreased by the addition of a more hydrophobic solvent.

Size exclusion chromatography (SEC)

SEC separates particles on the basis of size. It is generally low resolution chromatography and thus it is often reserved for the final “polishing” step of the purification. It is also useful for determining the tertiary structure and quaternary structure of purified proteins. This technique is widely used for the molecular weight determination of polysaccharides.

Procedure

The different columns were used in the following experiments:
BioSep SEC-S4000 (Phenomenex)
Dimensions: 300 x 7.8 mm
Synergi Max-RP (C12) Column (Phenomenex)
Dimensions 750 x 3 mm
BioBasic 18, 5 µm, (Thermoelectron)
Dimensions 150 x 4.6 mm
Sample preparations for injection:
Foot print of the starfish:
Starfish were placed in a dish in which glass slides were left at the bottom so that the starfish could walk over them. After 8 hours of collection, the slides were removed washed in distilled water and freeze-dried. 0.5 g of freeze-dried material was dissolved in 2 M urea, 0.1 M DTT, 0.063 M Tris pH 8 buffer. The extract was filtered with filter 0.45 µm filter (Millipore). A volume of 100 µl was injected onto the column.
Tube feet of starfish:
Tube feet were removed from the starfish and then 1g of the tube feet was homogenized with 3 ml buffer (2 M urea, 0.1 M DTT, 0.063 M Tris-HCl pH 8). The suspension was then centrifuged at 15000 x g for 15 min. The supernatant was filtered through a 0.45µm filter. A volume of 100 µl was injected on a column.

Various mobile phases were used depending on the type of column used:
Mobile phase for Biosep SEC-4000 column was 20 mM sodium phosphate, pH 7.2 (A)
Flow was isocratic (100% A) with a speed of 0.5 ml/min.

For column Synergi Max C-12, the mobile phase was: acetone and distilled water. A gradient method was used with various percentages of acetone and water (flow 1 ml/min). For Bio-Basic column the mobile phases were acetone and distilled water. Various gradients were set up for elution (flow 1 ml/min.).

4.2 Methods use for characterisation of adhesive material

4.2.1 Protein concentration assays

4.2.1.1 Bradford Assay

The Bradford dye-binding assay is a colorimetric assay for measuring total protein concentration (Bradford, 1976). It involves the binding of Coomassie brilliant blue to protein.

Procedure:

Bradford Reagent (Bio-Rad) was diluted with water in ratio 4:1. For each sample (25 µl) 1 ml of the diluted Bradford Reagent was added and incubated at room temperature for 5 min. Samples were placed in 96-well plates in duplicates (100 µl per well) and read. The absorbance was measured at a wavelength of 580 nm. If the concentration of the protein was unknown several samples of various dilutions were made. Protein standards were prepared in the same buffer as the samples to be assayed. A convenient standard curve was made using bovine serum albumin (BSA) with concentrations of 0, 62.5, 125, 250, 500 and 1000 µg/ml for the standard assay. Using protein standards, a graph of absorbance at 580 nm was prepared. The Bradford assay gives a hyperbolic plot for absorbance versus protein concentration, but within a range of relatively low protein

concentrations the hyperbolic curve can be approximated reasonably well by a straight line. The results are used to graph the standard curve from which unknown protein concentration are determined.

4.2.1.2 Lowry Assay, 1951 (2-D Quant Kit)

The assay is based on the specific binding of copper ions to protein. Precipitated proteins are re – suspended in a copper – containing solution and unbound copper is measured with a colorimetric agent. The colour density is inversely related to the protein concentration. The assay has a linear response to protein in the range of 0 – 50 µg.

The procedure uses a combination of a unique precipitant and co-precipitant to quantitatively precipitate sample protein while leaving interfering contaminants in solution. The protein is pelleted by centrifugation and re-suspended in an alkaline solution of cupric ions. The cupric ions bind to the polypeptide backbones of any protein present. A colorimetric agent which reacts with unbound cupric ions is then added. The colour density is inversely related to the concentration of protein in the sample. Protein concentration can be accurately estimated by comparison to a standard curve. Since the assay does not depend on reaction with protein side groups, it is largely independent of amino acid composition meaning that there is little variation in the response of this assay between different proteins.

Protein samples can be quantified with the 2-D Quant Kit directly in the electrophoresis sample solution. This simplifies sample preparation and assures that the protein assay result accurately reflects the protein concentration of the sample.

Procedure:

Bovine serum (2 mg/ml) was used to prepared standard solution. The tube had the following amounts of protein: 0 µg; 10 µg; 20 µg; 30 µg; 40 µg and 50 µg. Tubes containing 2 µl, 4 µl, 6 µl of the sample were prepared in duplicates. 500 µl the precipitant was added to each tube (including the standard curve tubes). The tubes were vortexed briefly and incubated 2-3 min at room temperature. 500 µl of the co-precipitant was added to each tube and mixed briefly by vortexing or inversion. The tubes were

centrifuged at a minimum of 10 000 x. g for 5 min resulting in a small pellet. The supernatants were decanted into 100 µl of copper solution and 400 µl of distilled water was added to each tube. Precipitated proteins were dissolved by vortexing. 1 ml of working colour reagent was added to each tube. Samples were mixed by inversion. The tubes were incubated at room temperature for 15 – 20 min. The absorbance of each sample and standard was read at 480 nm with water being used as the reference.

Unlike most protein assays, the absorbance of the assay solution decreases with increasing protein concentration. A standard curve was generated by plotting the absorbance of the standards against the quantity of protein. This standard curve was used to determine the protein concentration of the samples.

4.2.2 Measurement of adhesion

Equipment:

To measure adhesion, equipment was specially made for this experiment. This was based on an old type laboratory balance, modified by adding two blocks to it (made from either Teflon, iron, gelatine, glass, silicon). A block was attached to one beam of the balance while a second block attached to moving platform. Adhesion was measured as follows: a drop of measuring liquid (10 µl) was put on the block and stuck to the second block hanging from the beam. In this position, the drop of measuring liquid was incubated for 15 min at room temperature. To determine the adhesion of the liquid, standard masses were added until the two blocks separated when the adhesion of the liquid failed. The amount of weights put on to release the block and liquid was equivalent to adhesion forces between them.

Fig. 15 Equipment for measuring adhesion

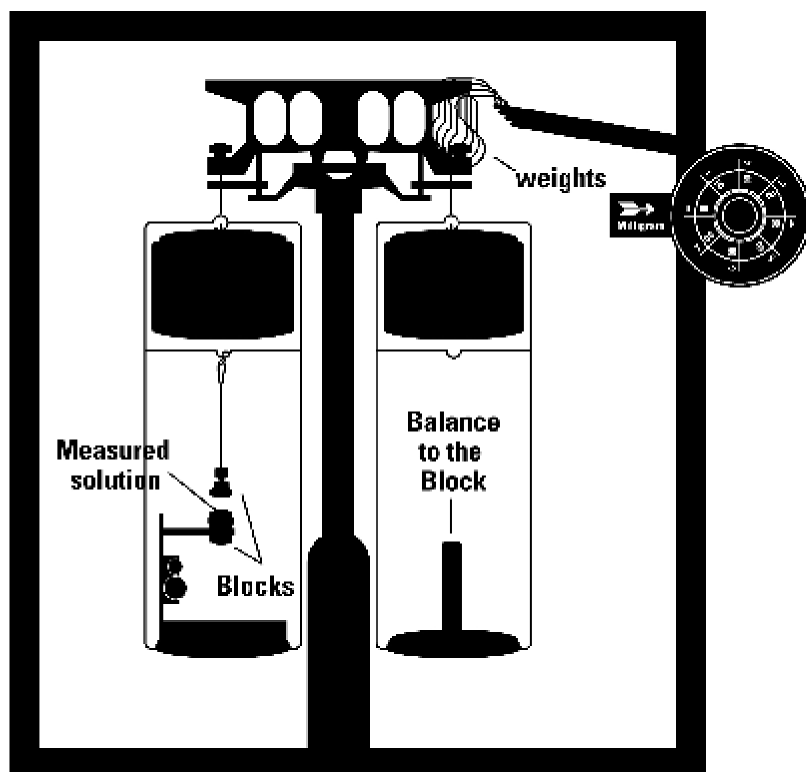


Fig. 16 Scheme of equipment for measuring of adhesion

A second apparatus for measuring adhesion was developed following of idea Müller and Zahn (1972).

The apparatus consisted of a sensitive spring balance; two microscope slides, one of which was attached to spring balance and the second covered with paraffin. A drop of liquid was put on the attaching slide and this was touched to the covered paraffin slide and incubated for 15 min. After this time, the spring balance was elongated by pulling, until finally the adhesive force suddenly failed, causing the drop to suddenly detach from the slide. The critical elongation was recorded.

Measurement of BSA adhesion (negative control)

A solution of 1 mg/ml BSA (bovine serum albumin) in 4 M urea, 0.5 M Tris buffer was used as a negative control. Various dilutions of BSA were tested. Buffer solution with a concentration equivalent to the measuring solution was used as a control. 10 µl of each sample was taken for measurement; each test was repeated three times.

Measurement of adhesion of Cell-Tak (positive control)

BD Cell-Tak adhesive is a formulation of the "polyphenolic proteins" extracted from the marine mussel *Mytilus edulis.* Cell-Tak is used to attach cells or tissue sections to many types of surfaces, including plastic, glass, metal and biological materials.

Cell-Tak was made up in 5% acetic acid to make the working solution. This was diluted with 6 M urea, 0.75 M Tris to obtain 4 M urea, 0.5 M Tris extract. Adhesion of the formulation in 5% acetic acid, 4 M urea, 0.5 M Tris pH 7.5 solution of Cell-Tak and 4 M urea, 0.5 M Tris buffer were measured. Experiment was repeated three times, statistical calculation of significance was made using Welch test (1977).

Measured adhesion on various surfaces

Extracts of Cuvierian tubules were used for the experiment. The extract was prepared using procedure 4.1.2.2. 4 M urea, 0.5 M Tris extract was diluted to several dilutions to obtain extract with 2 M urea, 1 M urea, 0.5 M urea, 0.2 M urea, 0.1 M urea.
4 M urea, 0.5 M urea buffer was also diluted to these same dilutions. Buffer was used as a negative control in all measurement.

10 µl of each sample was taken for each measurement. Surfaces used for testing were: Teflon, silicone, glass, iron and gelatine. Experiment was repeated three times, statistical calculation of significance was made using Welch test (1977).

Impact of protein concentration on adhesion

Extracts of Cuvierian tubule were used for the experiment. The extract was prepared using procedure 4.1.2.2. 4 M urea extract was diluted to obtain 0.5 M urea extract.

0.5 M urea extract was diluted with 0.5 M urea buffer to obtain various concentrations of protein. 10 µl of sample was taken for each measurement and the measurement was repeated three times. The average of the results was used for analysis. 0.5 M urea buffer was used as a negative control.

Impact of metal cations and EDTA on adhesion

Extracts of Cuvierian tubules were used for this experiment. The extract was prepared using procedure 4.1.2.2. 4 M urea extract was diluted to obtain 0.5 M urea extract.

Solutions of extract with the following concentration of cations and EDTA were prepared: 50 mM K^+,100 mM K^+, 5 mM Ca^{2+}, 5 mM Mg^{2+}, 5 mM Cd^{2+}, 5 mM Zn^{2+}, 10 mM EDTA

0.5 M urea buffer used for calibration of the equipment and as a negative control. 0.5 M urea extract was used as the positive control. 10 µl of the each solution was used for each measurement and each sample was measured three times. The average of results was used for analysis. Statistical calculation of significance was made using Welch test (1977).

Measurement of adhesion of fractions from gel filtration

Fractions obtained in gel filtration 4.1.10 were taken for measurement. Six fractions which contained the highest concentration of the protein were measured.

10 µl of each fraction was taken for measurement. The measurement was repeated five times and the average of results was used for further analysis. 4 M urea buffer was used to calibrate the equipment and as a negative control. 4 M urea extract was used as a positive control. All the samples were incubated for 15 min at room temperature during

the measurement. Statistical calculation of significance was made using Welch test (1977).

Measurement impact on adhesion by cations and EDTA on fractions from gel filtration

Each fraction eluted from the gel filtration which contained adhesive protein was tested for the effect on adhesion of K^{+} (50 mM concentration), Ca^{2+} (5 mM), Zn^{2+} (5 mM), EDTA (10 mM) and Cd^{2+} (5 mM). Experiment was repeated three times, statistical calculation of significance was made using Welch test (1977).

Adhesion of extract neutralized by antibody

Extract of Cuvierian tubules and antibody against the adhesive protein were used for this experiment. The extract was prepared using procedure 4.1.2.2. The antibody was prepared using procedure 4.1.6.

600 µl of the 4 M urea extract was added to 5 ml of distilled water to obtain 0.5 M urea buffer solution. 5 ml of 0.5 M urea extract was mixed with antibody (10 µl) and left in room temperature for 1 h. This procedure was used to neutralize the adhesive protein.
0.5 M urea buffer was used to calibrate the equipment (negative control). A drop of the buffer (10 µl) was put onto slide and then stuck to the other slide and incubated for 15 min. After this time, the adhesion force was measured. The experiment was repeated three times and the average of the results was taken. 0.5 M urea extract was used as the positive control. A 0.5 M mixture of antibody and extract was measured with same scheme as above. All results were compared and analysed. Statistical calculation of significance was made using Welch test (1977).

Trypsin

Trypsin is a serine protease found in the digestive system, where it breaks down proteins. This enzyme predominantly cleaves peptide chains at the carboxyl side of the amino acids lysine and arginine, except when they are followed by proline.

Trypsin and the extract of Cuvierian tubules was mixed (1:1 and 1:2) and incubated for 15 min at room temperature. After this time, adhesion of the mixture was measured. Buffer with the same composition of solvents was used as a control. Experiment was repeated three times, statistical calculation of significance was made using Welch test (1977).

Adhesion of mussels extract

Mussels *Mytilus galloprovincialis* were removed from the shell and homogenised with 4 M urea, 0.5 M Tris pH 7.5. After grinding with a mortar, the suspension was centrifuged (15 min, 5000 x g). The supernatant was removed and measured in the same way as that of the extract from the Cuvierian tubules. Various dilutions were made to determine how adhesion was dependant on the concentration of the urea and protein.

Experiment was repeated three times, statistical calculation of significance was made using Welch test (1977).

Adhesion of silk fibroin

The adhesive properties of isolated recombinant protein silk fibroin were measured. Elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole) was used as a negative control. A 10 µl of extract of Cuvierian tubules was used as the positive control. Experiment was repeated three times, statistical calculation of significance was made using Welch test (1977).

4.2.3 Preparations of slide sections

Tissue was fixed in 2% paraformaldehyde in phosphate buffer (pH 7.4 overnight). The next step was overnight washing in PBS buffer (pH 7.4) containing 6.8% sucrose at 4 °C. The tissue was dehydrated in 100% acetone. During the first 5 min acetone was renewed a few times until the solution remained clear. The last portion of acetone was kept overnight. The infiltration of the samples was done using a Technovit 8100 (Heraeus Kulzer) according to the instructions of the manufacturer. The infiltration solution consists of Technovit 8100 (100 ml base-liquid) and 1 bag hardener I (0.6 g). The tissue to be embedded is immersed in the solution (15 ml infiltration solution and 0.5 ml hardener) and agitated for 5 min. The well-mixed embedding solution was poured into the embedding mould and the tissue specimen was orientated. Immediately afterwards, the mould was hermetically sealed with cover foil and placed on crushed ice at 4°C.

Fixing of embedded specimens was done in Histobloc using Technovit (mixed in a ratio of 2 parts by volume powder to 1 part by volume liquid) that was poured into the recess in the back of the Histobloc up to a level of about 2 mm above the base of the Histobloc. After 5 – 10 min the specimen was removed from the mould. Sections of 3 – 10 µm thickness were prepared through the use of a rotary microtome and slices were mounted on silane-coated slides to avoid adhesion-problems.

4.2.4 Immunohistochemistry

Immunohistochemistry (IHC) refers to the process of localizing proteins in cells of a tissue section exploiting the principle of antibodies binding specifically to antigens in biological tissues. Immunohistochemical staining is widely used in clinical diagnostics and in laboratory research for understanding the distribution and localization of proteins in different parts of a tissue.

Procedure:

The prepared sections were kept overnight in 4% bovine serum albumin in PBS. They were then washed 2 x 10 min in PBS after blocking. The samples were incubated with polyclonal antibody (poAb no. N384, 1:100)

Slides with antibody against adhesive protein were kept for 2 hours at room temperature in a humidity chamber. After washing 2 x 10 min in PBS, the slides were incubated with Cy3-conjugated goat anti-rabbit IgG (diluted 1:100) for 120 min in the dark on 37ºC. After additional washing of 2 x 10 min in PBS staining with DAPI (4'-6-Diamidino-2-phenylindole) for 30 min was done in order to highlight the nuclei. After washing and mounting with the gel/mount, hardened (Dako) nail polish was put around the cover slip to avoid the samples drying out irregularly and the sections were inspected with an Olympus AHBT3 fluorescence light microscope. Pre-immune serum was used as a negative control.

4.2.5 Stained slides

4.2.5.1 Haematoxylin & Eosin

Haematoxylin and Eosin is a popular staining method in histology. This staining method involves application of the basic dye Haematoxylin, which colours basophilic structures with blue-purple hue, and alcoholic-based Eosin Y, which colours eosinphilic structures bright pink.

Procedure:

Slides were washed with PBS for 5 min. They were then put in distilled water for 5 min followed by Haemotoxylin staining for 5 min and washed in tap water for 25 min, then in distilled water for 5 min. They were then stained in Eosin for 1 min. The next step was a short wash in distilled water. The slides were dehydrated in alcohol with increasing concentration (75 – 100%) for 1 min in each step. Before mounting, the slides were washed in a detergent (Roti Histoclear-which is based on xylol) 3 times for 5 min. Slides were mounted in DPX (Sigma), covered with cover slips and sealed with nail polish.

4.2.5.2 Alcian blue, PAS

This is a combined method utilising the properties of both the PAS and Alcian Blue methods to demonstrate the full complement of tissue proteoglycans.

The rationale of the technique is that by first staining all the acidic mucins with Alcian blue, those remaining acidic mucins which are also PAS positive will be chemically blocked and will not react further during the technique. Those neutral mucins which are solely PAS positive will subsequently be demonstrated in a contrasting manner. Where mixtures occur, the resultant colour will depend upon the dominant moiety.

Procedure:
Sections on slides were washed with distilled water. The slides were then stained with Alcian blue for 15 min. After this time slides were washed thoroughly with tap running water for 5 min. The slides were then treated with periodic acid (PAS) for 5 min. After staining slides were washed with distilled water. Slides were then stained with Schiff's reagent (10 min) and Haemotoxylin (1 min). They were then washed with distilled water and differentiated with acid alcohol (1% acetic acid in ethanol) and dehydrated in increasing concentrations of alcohol and washed in detergent before mounting with DPX.

4.2.5.3 Coomassie Blue

Coomassie dye stains a wide range of proteins**.** This stain is usually used for polyacrylomide gels but can also be used to stain sections on slides**.** Coomassie dye stains proteins very strongly blue. The Coomassie dye binds to proteins via physisorption to arginine, the aromatic amino acids, and histidine.

Procedure:

Slides were washed with distilled water for 5 min. After washing slides were stained for 15 min in 1% Coomassie Blue, then washed with water and dehydrated with alcohol before being cleared in detergent (Roti Histoclear). Slides were mounted with DPX and sealed with nail polish.

4.2.5.4 Alcian blue, Ponceau, Acid fuchsine

Alcian blue is a group of polyvalent basic dyes that are water soluble. The blue colour is due to the presence of copper in the molecule. In a 3% acetic acid solution (pH 2.5), Alcian blue stains both sulfated and carboxylated acid mucopolysaccharides and sulfated and carboxylated sialomucins (glycoproteins). It is believed to form salt linkages with the acid groups of acid mucopolysaccharides. Acid fuchsine stains proteins (cytoplasm and membranes), collagen-pink, Ponceau stains fibrin red.

Procedure:

Stains were prepared as follows:

0.167 g of fuchsine acid and 0.667 g of Ponceau were mixed in 500 ml of water and 1 ml of acetic acid. 1 g Alcian blue was dissolved in 100ml of 3 % acetic acid and pH was adjusting to 2.5. Starfish were allowed to adhere to slides for 1 hour. The slides were then gently washed with distilled water.

Footprints on the slides were fixed with 10% formalin. The slides were stained with Alcian blue for 15 min. After this time, the slides were washed with distilled water three times for 2 min, before being stained in the Ponceau-fuchsine mixture for 10 min. The next step was washing in 1% acetic acid and dehydrated in alcohol. Slides were mounting with DPX and sealed with nail polish.

4.2.5.5 Casson’s Trichrome

This stain is usually used for staining muscle and collagen. It is composed of four various stains, each stain is responsible for differentiating a particular part of tissue or cell (nuclei (stains red), erythrocytes (orange), cytoplasm (red), collagen (blue))

Casson’s Trichrome contains:

1% Orange G

1.5% Acid fuchsine

0.5% Aniline blue

1% Phosphotungstic acid

Procedure:

Slides were washed in PBS for 5 min. Sections were placed in staining solution for 5 min. After staining, slides were washed in water for 5 min. The next step was dehydrating slides in various alcohol solutions with increasing concentration of alcohol.

The slides were cleared in detergent (Roti Histoclear), mounted with DPX and sealed with nail polish.

4.2.5.6 Methylene blue – Azure B

Azure, a methylated thiazine dye, is a metachromatic basic dye, producing a variety of colours ranging from green (to chromosomes) and blue (to nucleoli and cytoplasmic ribosomes), to red colour (to deposits containing mucopolysaccharides).
Methylene blue is a homologue of Toluidine blue O. This stain is used to visualize intracellular metachromatic granules. By this method, the intracellular metachromatic granules stain a ruby-red to black colour; with the remainder of the cell staining a pale blue colour.

Procedure:

A mixture of 1% Azure and 1% Methylene blue were used for staining. Before staining sections were warmed up, up to 70 ºC on Heatplate. A drop of the stain was put on the sections then they were dried at 60 ºC. Unbound stain was then washed out distilled water and dried. Slides were mounted with DPX and sealed with nail polish.

4.2.5.7 Haematoxylin & Oil Red O

The Oil Red O stain is commonly used to identify exogenous or endogenous lipid deposits. The stain is applied to frozen sections of fresh or formalin-fixed tissue. Haematoxylin stains nuclei on blue, Oil Red stains lipid on red for lipid or fat staining. These composition of stains helps identify lipid in section.

Procedure:

A small amount of propylene glycol was added to the 0.5 g Oil Red O and stirred. To the mixture was gradually added the remainder of the propylene glycol. The mixture was heated until the solution reached 95 ºC. The stain was filtered through coarse filter paper while still warm. The solution was left overnight at room temperature. Slides were air dried for 30 minutes, and then fixed in ice cold 10% formalin for 5 minutes. Slides were rinsed in three changes of distilled water then placed in absolute propylene glycol for 5 min to avoid carrying water into the Oil Red O. The slides were then stained in Oil Red O solution for 8 min in a 60 ºC oven and then rinsed in 85% propylene glycol solution for 5 min. The next step was a rinse in distilled water. Sections were stained in Haemotoxylin for 30 sec. The slides were then washed thoroughly in running water for three min and rinsed in two changes of distilled water. Sections were mounted with DPX and sealed with nail polish.

4.2.5.8 Toluidine blue

Toluidine blue stain mast cells red-purple (metachromatic staining) and the background blue (orthochromatic staining). Metachromasia, (tissue elements staining a different colour from the dye solution), is due to the pH, dye concentration and temperature of the basic dye. Blue or violet dyes should show a red colour shift, and red dyes will show a yellow colour shift with metachromatic tissue elements.

Procedure:

Sections were stained in Toluidine blue working solution (5 ml of Toluidine blue stock solution, 45 ml of 1% sodium chloride, and pH 2.3) for 3 min. Slides were washed three times with distilled water then dehydrated through 95% and two changes of 100% alcohol. Slides were cleared in xylene and mounted with DPX.

4.2.6 AFM

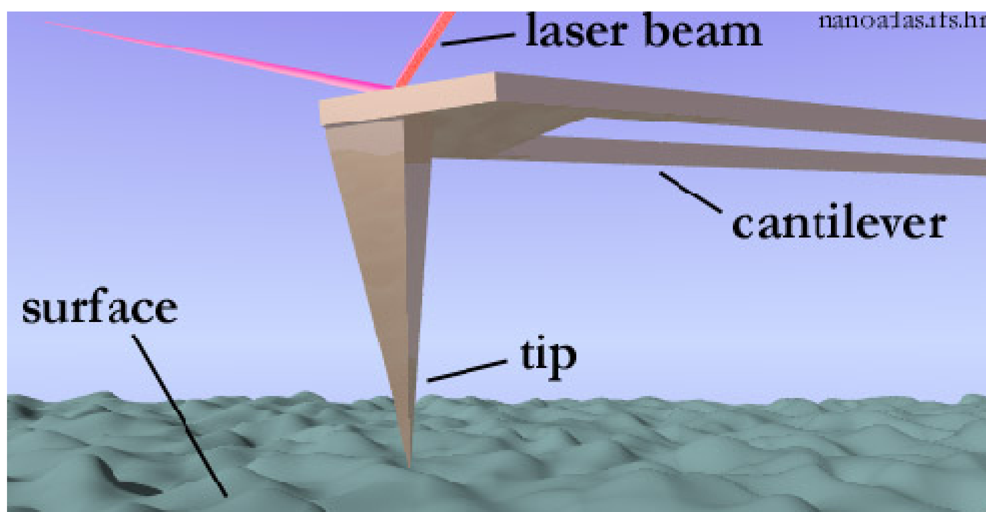
The atomic force microscope (AFM) is a new type of scanning probe microscope which is capable of mapping forces near surfaces or, by means of these forces, mapping the

topography of the surface. The AFM is an offshoot of the scanning tunnelling microscope (STM). Like the STM, the AFM is capable of extremely high spatial resolution in three dimensions. The AFM, however, can be used to study the surfaces of both electrically conducting and non conducting samples, overcoming a major limitation of the STM.

The AFM probe typically has a sharp tip (5-50 nm radius of curvature) making the contact area of tip-substrate quite small. Attractive and repulsive forces between the AFM tip and the substrate at sub-nano Newton level can be monitored. For high resolution measurements, the sample is usually scanned instead of the tip as with the STM (i.e. the sample is moved rather than the tip), because the AFM measures the relative displacement between the cantilever and sample surfaces and any cantilever movement would add vibrations. For measurements of large samples, AFMs are available where the tip is scanned and the sample is stationary.

Contact mode AFM operates by scanning a tip attached to the end of a cantilever across the sample surface while monitoring the change in cantilever deflection with split photodiode detector.

Tapping mode AFM operates by scanning a tip attached to the end of an oscillating cantilever across the sample surface. The cantilever oscillates at or near its resonance frequency with the amplitude ranging typically



from 20 nm to 100 nm.

Fig.17 Scheme of imaging using AFM

Procedure:

A Bioscope I with dimension head (single piezo) and Nanoscope 3a controller were used for all experiments. Fresh footprint of the starfish was used for the measurement of adhesive forces.

Starfish adhered to the slides and after a few hours a useful number of footprints were obtained. Some of the slides were washed in distilled water and some of them were washed with filtered sea water and left to dry. Contact mode in dry conditions was used for measurement of adhesion. The type of tip used was the DNP-S- 20.

Fixed footprints were used for characterisation.

Footprints were fixed in 10% formalin and washed in distilled or filtered sea water. Tapping mode in wet condition was used for characterisation of the surface of the footprint. Solvents uses for tapping mode were ultrapure water or filtered sea water.

4.2.7 Wyko NT 1100

The Wyko NT1100 apparatus (owned by VEECO Cambridge) provides non-contact imaging at high speed with a large field of view. Fast and repeatable, the NT 1100 provides high resolution 3D surface measurement, from sub-nanometer roughness to millimeter-high steps. The NT 1100 is used for thick films, optic, ceramics and advanced materials and uses a process of interferometry. Interferometry is a versatile measurement

technology for examining surface topography with very high precision. At the heart of interferometry is the interfeogram which is the recorded interference signal of two beams of light existing from the same source. An interferogram carries a wealth of information about the profile of an object under test and its material characteristic.

Images of the footprint scan by Wyko in dry conditions have helped determine the structure of the footprint. Data obtained from the Wyko was analysed by the Vision 32 analytical software package. Advanced optics in the Wyko ensured sub-nanometer vertical resolution at all magnifications. The Data Stitching option added a motorized stage for high resolution measurements over a larger field of view.

Procedure

Starfish were allowed to adhere to the slides. After a few hours the slides had large numbers of the footprints after adhesion of the starfish. Slides were washed in distilled water to remove any salt residues. Footprints were fixed in 10% formalin for 10 min.

Slides were washed in distilled water and dried. A coverslip chamber was put on the slides to prevent any damage of the footprint. Slides were sent to Veeco Cambridge where technicians did the measurement.

4.2.8 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) is a microscopy technique whereby a beam of electrons is transmitted through an ultra thin specimen, interacting with heavy metals that are used to stain and thus differentiate the specimen as it passes through it. A negative image is formed from the electrons transmitted through the specimen, magnified and focused by an objective lens and appears on a fluorescent screen in most TEMs, plus a monitor, or on a layer of photographic film, or to be detected by a sensor such as a CCD camera.

Procedure:

Electron immunogold labeling was performed with Cuvierian tubules samples treated in 0.1% glutaraldehyde in 3% paraformaldehyde buffered in phosphate buffer, pH 7.4 for 3h at room temperature. The material was dehydrated in ethanol and embedded in LR-White resin. 60 nm thick slices were cut and blocked with bovine serum albumin in PBS and then incubated with the primary antibody poAb against adhesive protein from Cuvierian tubules (no. N384; 1:1, 00 for 12 h at 4 °C). In controls, preimmune serum was used. After three washes with PBS, 1 % BSA, sections were incubated with a 1:100 dilution of the secondary antibody (1.4 nm nanogold anti-rabbit IgG; diluted 1:200) for 2 h. Sections were rinsed in PBS, treated with glutaraldehyde in PBS, washed, and dried. Subsequently, enhancing of the immunocomplexes was performed with silver as described Danscher (1981). The samples were transferred onto coated copper grids and analyzed with a Tecnai 12 microscope (FEI Electron Optics, Eindhoven; Netherland).

5. Results

The main goal in this research was to isolate and characterise adhesive protein from two species of echinoderms (*Holothuria forskali* and *Marthasterias glacialis* drawn from two classes: the Holothuroidea and the Asteroidea).

The research consisted of two broad steps. In the first proteins were isolated from the specimens by a wide range of methods including: electro-elution, immunoprecipitation, digestion and HPLC. The second step was characterisation of the adhesive proteins by methods such as immunohistochemistry, AFM and Wyko.

5.1 SEA CUCUMBER *HOLOTHURIA FORSKALI*

5.1.1 Isolation and identification of adhesive protein from Cuvierian tubules

5.1.1.1 Extraction of Cuvierian tubules

Among the conditions that were tested to extract Cuvierian tubules, protein solubilisation was improved in basic rather than acidic buffers. Urea, SDS, and reducing agents improved the extraction of Cuvierian tubules proteins. The amount of proteins extracted using various buffers were compared on an electrophoresis gel. The best result was obtained using 4 M urea, 0.5 M Tris in pH 7.5 (Fig. 18, lane 3) and sample loading buffer (Fig. 18, lane 1). The 150 mM NaCl, 1.5% NP_{40}, 0.1% SDS, 0.1% DOC, 50 mM Tris pH 8 buffer and the 0.5 M NaCl, 5 mM Tris, 7 mM Na_2SO_4, 0.4 mM $NaHCO_3$, 20 mM EDTA buffer (Fig. 18, lane 2 and 4) did not solubilise enough of the tissue to see strong bands on the SDS-PAGE. Urea buffer was used for further analysis because the composition of the sample loading buffer (100 mM Tris, pH 6.8, 2% SDS, 5% ß-mercaptoethanol, 15% glycerol, 0.006% bromophenol blue) may have caused problems with some of the tests to be performed.

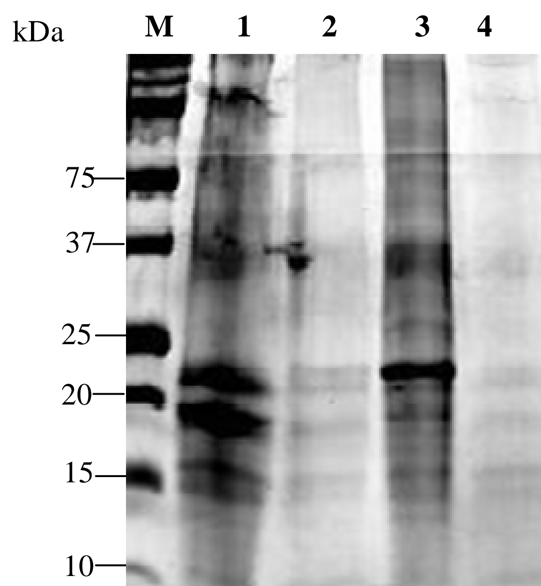


Fig. 18 Extraction of Cuvierian tubules with different buffers, analysis on 12% SDS-PAGE gel, stained with GelCode Blue Reagent (**1** – sample buffer (100 mM Tris, pH 6.8, 2% SDS, 5% ß-mercaptoethanol, 15% glycerol, 0.006% bromophenol blue), **2** – 0.5 M NaCl, 5 mM Tris, 7 mM Na_2SO_4, 0.4 mM $NaHCO_3$, 20 mM EDTA **3** – 4 M urea, 0.5 M Tris and **4** – 150 mM NaCl, 1.5% NP_{40}, 0.1% SDS, 0.1% DOC, 50 mM Tris pH 8, **M** – marker)

The concentration of protein in Cuvierian tubules extract was estimated by Bradford assay to be approximately 1 mg/ml. After staining of the proteins with Gel Code Blue Reagent a smear of proteins was observed in the gel (Fig. 19 A), which made impossible to visualise bands. As a consequence a purification of the extract was done using Ready Prep™ 2-D Clean-up kit. Electrophoresis gels of the purified Cuvierian tubules extract showed the presence of a wide size range of proteins (Fig. 19 B). Gels stained with Gel Code Blue Reagent showed few strong bands and many weak bands. There are both small proteins of around 15-20 kDa and larger ones (50 kDa – 200 kDa) present. To get more information about the conformation and the dimerisation of the proteins, the extract was run on a semi-native gel (Fig. 19 C). The results showed that the number of the bands was reduced, indicating that some proteins are present in dimers.

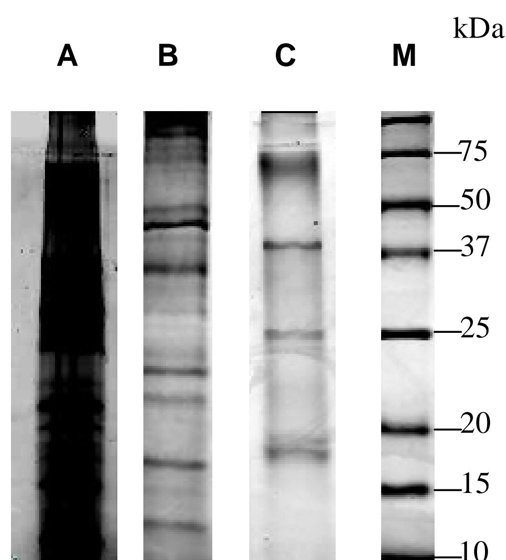


Fig. 19 Analysis of Cuvierian tubules (CT) extract from sea cucumber *Holothuria forskali* in 4 M urea, 0.5 M Tris pH 7.5 by electrophoresis gel, stained with GelCode Blue (**A** – CT extract on 12% SDS Page gel, without prior purification, **B** – CT extract loading on 12% SDS-PAGE gel after purification with Ready Prep^{TM} -2D Clean up kit, **C** – CT extract, loading on semi-native gel, **M** – marker)

5.1.1.2 Identification of adhesive protein in Cuvierian tubules

An antibody against an adhesive protein from mussels (recombinant protein silk fibroin) was used to identify molecular weight of the adhesive protein found in Cuvierian tubules. After comparing the sequence of gene of silk fibroin with the sequence of genes from echinoderms using the computer programme BLAST, 40% similarity was found in the sea urchin *Strongylocentrotus purpuratus*, meaning that antibody against silk fibroin could be used to detect protein in echinoderms. After reaction with the antibody, the membrane showed a strong band (Fig. 20, lane B) with an 18 kDa protein from the Cuvierian tubules. This protein was recognised as the protein involved in adhesion in further research (see below). Silk fibroin was used like positive control was this antibody (Fig. 20, lane A).

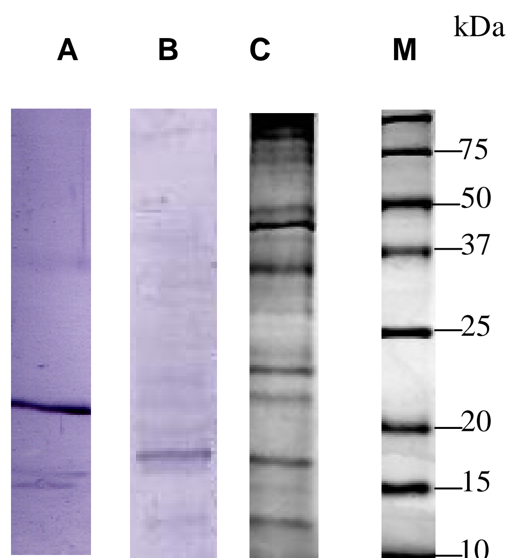


Fig. 20 Identification of adhesive protein in extract of Cuvierian tubules from sea cucumber *Holothuria forskali* using antibody against silk fibroin, adhesive protein in mussel *Mytilus galloprovincialis* (**A** – Western blot of silk fibroin, adhesive protein from mussel, incubated with antibody against it, positive control, **B** – Western blot CT extract, incubated with antibody against silk fibroin (1:1000 dilution) and developed with anti-rabbit IgG alkaline phosphatase, **C** – 12% SDS-PAGE of CT extract, **M** – marker)

5.1.1.3 Isolation of identified protein

5.1.1.3.1 Electro-elution

After highlighting the adhesive protein, the next step was its isolation. The band with a molecular weight of 18 kDa was cut off from gel and electro-eluted. Around 2 mg of all extracted protein was necessary to obtain a concentration of adhesive protein of 600 μg/ml. However the concentration of the purified protein was determined by Lowry assay instead of Bradford, because the obtained protein was in Coomassie blue solution which could interfere with the protein measurement. Protein was injected to the rabbit and after 4 weeks, the specific antibody was obtained.

5.1.1.3.1.1 Neutralisation of antibody

After loading the crude extract of the Cuvierian tubules in a Western blot, the antibody showed a strong reaction with many proteins of the extract even under optimised concentration of the antibody and the extract (Fig. 21 C).

The antibody was neutralised (captured antibody) with the full Cuvierian tubules extract and incubated for 1 – 2 hours. After binding the extract mixture was applied to the membrane of a Western blot. Excess of specific antibody in the mixture bound to specific antigens on the membrane.

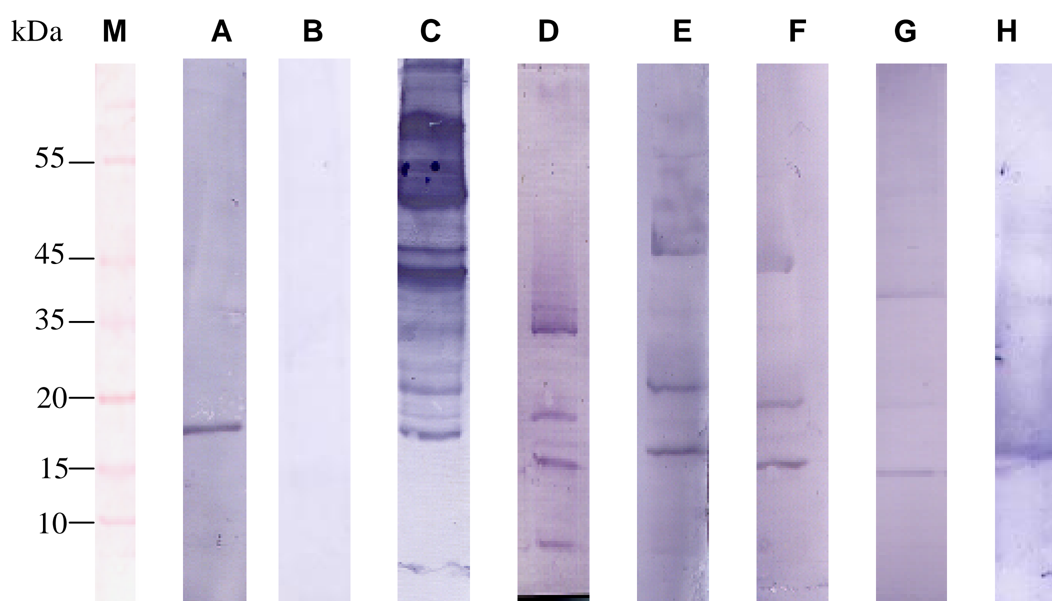


Fig. 21 Developing optimal condition for using antibody against adhesive protein from Cuvierian tubules (CT) of sea cucumber *Holothuria forskali* (**M** – marker, **A** – Western blot of adhesive protein, positive control, **B** – Western blot CT extract incubated with preimmune serum against adhesive protein, **C** – Western blot of CT extract incubated with antibody against adhesive protein dilution 1:1000, **D** – Western blot CT extract incubated with antibody against protein, dilution 1:1000 with 1:10 extract, **E** – Western blot of CT extract incubated with antibody against adhesive protein dilution 1:1000, with 1:5 extract, **F** – Western blot of CT extract incubated with antibody against adhesive protein dilution 1:2000, with extract 1:5, **G** – Western blot of CT extract incubated with antibody against adhesive protein dilution 1:1000 with extract 1:5, loading extract diluted 1:10, **H** – Western blot of CT extract running on semi-native gel, dilution 1:1000 with 1:5, extract diluted 1:10

Maximum specification could be observed with increasing amount of extract in the antibody mixture. The best results were obtained with an antibody dilution of 1:1000 with addition of extract at 1:5 (Fig. 21 G). The Western blot showed that two proteins reacted with the antibody, one with a molecular weight of 18 kDa and the other of 36 kDa. It was possible these two proteins could actually be a dimer. In order to verify if that was the case, a native gel was done and after transferred to the membrane and then incubated with the antibody against the adhesive protein. The membrane showed only a single band confirming the dimer conformation of the adhesive protein (Fig. 21 H).

5.1.1.3.2 Immunoprecipitation

The amount of adhesive protein isolated by electro-elution was very low and the buffer interfered with the protein analysis. Other methods for isolation were developed to overcome these problems.

Immunoprecipitation resulted in a low yield of adhesive protein obtained by this method was very low, only a weak band was observed in Western blot (Fig. 22 A).

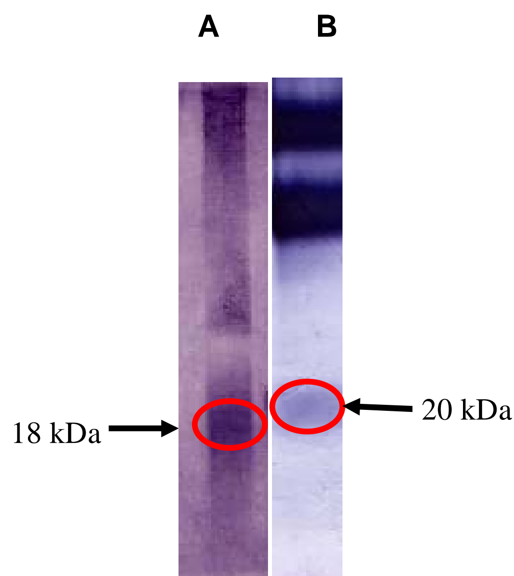


Fig. 22 Western blot analysis of adhesive protein purified by immunoprecipitation. The blot was developed using antibodies against adhesive protein from CT and anti-rabbit IgG alkaline phosphatase antibody as a secondary antibody (**A** – adhesive protein (red circle) incubated with antibody against protein from CT, **B** – protein from mussels (silk fibroin, red circle), incubated with antibody against adhesive protein from CT).

Isolated adhesive protein was also tested with the antibody against silk fibroin from mussels but there were no satisfactory results, possibly as the concentration of the obtained protein was too low to detect by antibody. In parallel, mussels extract was applied on the immuno-column with antibody against the adhesive protein from Cuvierian tubules. The result is shown on Fig.22 B, with a weak band around 20 kDa being observed.

5.1.1.3.3 Gel filtration

All the fractions after gel filtrations were assayed using Bradford assay to detect the presence of proteins. Fractions which contained proteins were loaded on an electrophoresis gel.

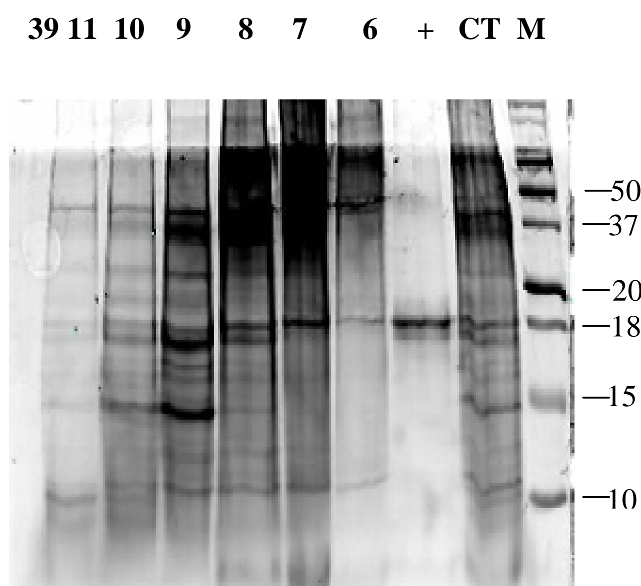


Fig. 23 Visualisation results of gel filtration column by 15% SDS-PAGE (**39** – fraction with low concentration of protein (negative control), **6....11** – fractions with highest concentration of protein after Bradford assay, ± positive control adhesive protein obtained by electro-elution from CT extract, **CT** – extract of Cuvierian tubules in 4 M urea, 0.5 M Tris buffer pH 7.5, **M** – marker)

The presence of adhesive protein could be observed in fractions 6, 7, 8. In fractions 7, 8 there were some contaminations from high molecular weight proteins. Their molecular weights are determined using the formula:

$\text{Log } M = M_0 - (6.062 - 5.00 * d) (V_e/V_0)$ (Determann, 1969)

M – Molecular weight, **d** – density of the swollen gel**,** $\mathbf{V_e}$ – elution volume, $\mathbf{V_0}$ – void volume

The Sephadex G-50 column used in this experiment conforms to:

$\text{Log } M = 5.189 - 0.712 (Ve/ V_0)$ (Muller *et al.,* 1973).

Molecular weights for the fractions containing protein were:

Fraction 6: 21,076 ± 5,955 Daltons, Fraction 7: 15, 121 ± 4,272 Daltons

Fraction 8: 10, 849 ± 3, 066 Daltons, Fraction 9: 7.783 ± 2,199 Daltons

Fraction 10: 5, 584 ± 1,578 Daltons, Fraction 11: 4, 006 ± 2, 874 Daltons

5.1.1.3.4 Glass beads

Various sizes of the beads

The results showed that the adhesion increased with the sizes of the beads. Results are expressed as a mean ± SD with a number of samples in parenthesis.

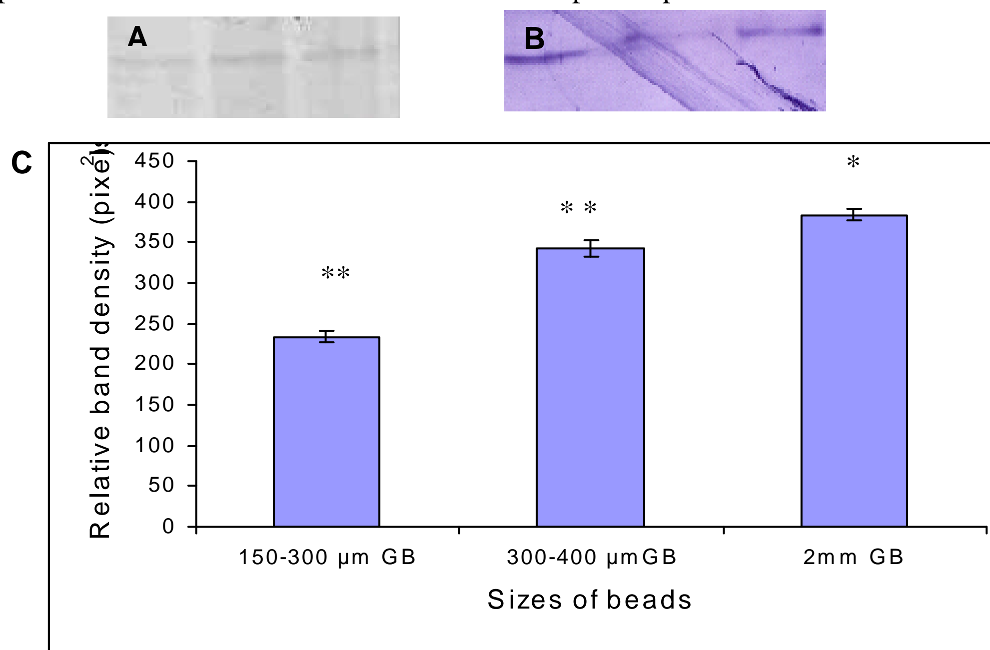


Fig. 24 Correlations between various sizes of glass beads and amount of isolated adhesive protein from the extract Cuvierian tubules (**A** – electrophoresis gel of fractions obtained from isolation of adhesive protein with various sizes of beads, **B** – Western blot of fractions obtained from isolation of adhesive protein with various sizes of beads from elution, **C** – diagram of correlation between various sizes of glass beads and amount isolated protein) $*P < 0.05$, $* * P < 0.01$, $*** P < 0.001$ (Welsch, 1977

Various concentration of urea

Results after testing several dilution show that dilution of extract was increasing adhesion of the protein. Results are expressed as a mean ± SD with a number of samples in parenthesis.

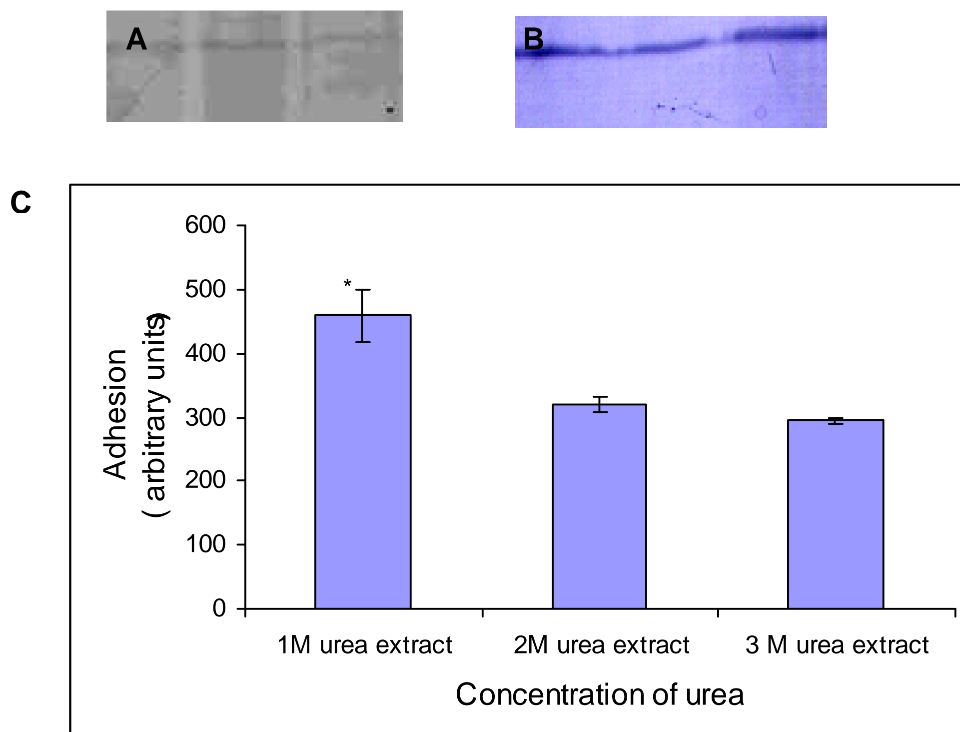


Fig. 25 Correlations between various sizes of glass beads and amount of isolated adhesive protein from the extract CT (**A** – electrophoresis gel of fractions obtained from isolation of adhesive protein with various concentration of extract**, B** – Western blot of fractions obtained from isolation of adhesive protein with various concentration of protein, **C** – Diagram of correlation between various sizes of glass beads and amount isolated protein). *P < 0.05, * * P < 0.01, *** P < 0.001 (Welsch, 1977)

5.1.2 Characterisation of adhesive protein in Cuvierian tubules

5.1.2.1 Histological identification

Cuvierian tubules were stained with Casson's Trichrome, Haemotoxylin & Eosin and Methylene blue & Azure stains to study their structure.

Staining with above mentioned stains showed that the structure of quiescent tubules *Holothuria forskali* consist of an outer mesothelium and an inner epithelium

encompassing a thick connective tissue layer. The outer part of the connncective tissue is made up of muscle fibres. Tubules have a pseudostratified mesothelium made of two cell layers, viz. an upper layer of adluminal cells and a lower layer of granular cells.

Casson' Trichrome

In section of Cuvierian tubules stained with Casson's Trichrome, mesothelium was observed stained red and the inner connective tissue stained blue (Fig.26).

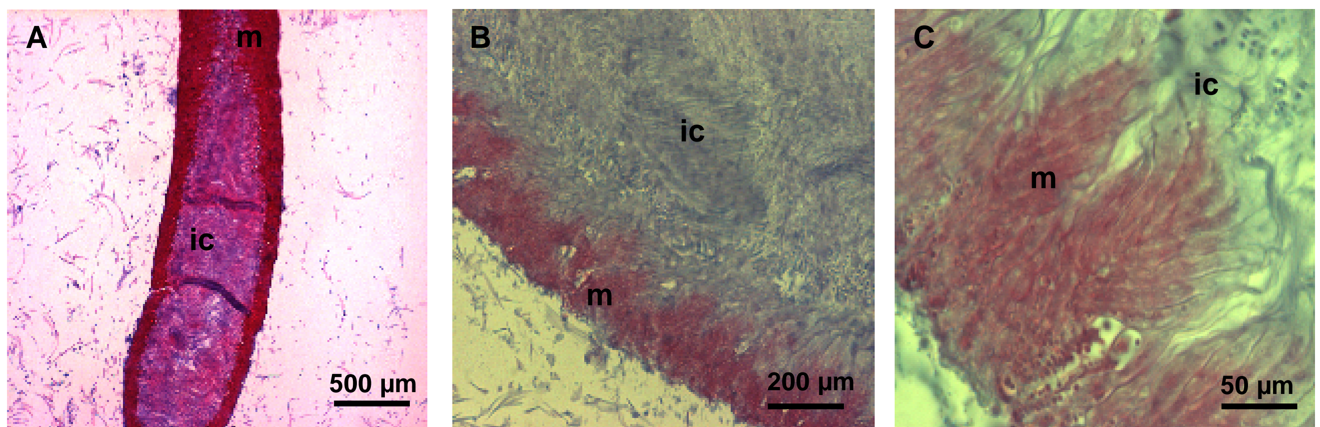


Fig. 26 Sections of Cuvierian tubules stained with Casson's Trichrome (**A** – 40 x magnification, **B** – 100 x magnification, **C** – 400 x magnification; **m** – mesothelium, **ic** – inner connective tissue)

Haematoxylin & Eosin

In sections stained with Haemotoxylin & Eosin, it was possible to observe inner connective tissue layer stained red with Eosin, while in the mesothelium the granular cells were stained purple (Fig. 27).

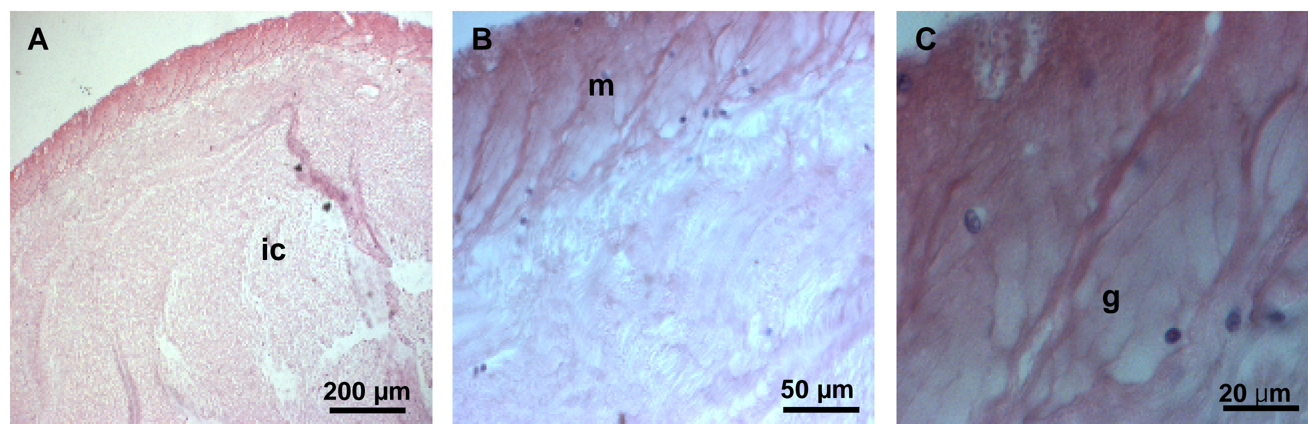


Fig. 27 Sections of Cuvierian tubules stained with H&E (**A** – 100 x magnification, **B** – 400 x magnification, **C** – 1000 x magnification; **ic** – inner connective tissue, **g** – granular cells)

Methylene blue & Azure B

Section stained with Methylene blue & Azure B showed the presence of blue mesothelium and red inner connective tissue, while granular cells were stained with black (Fig. 28)

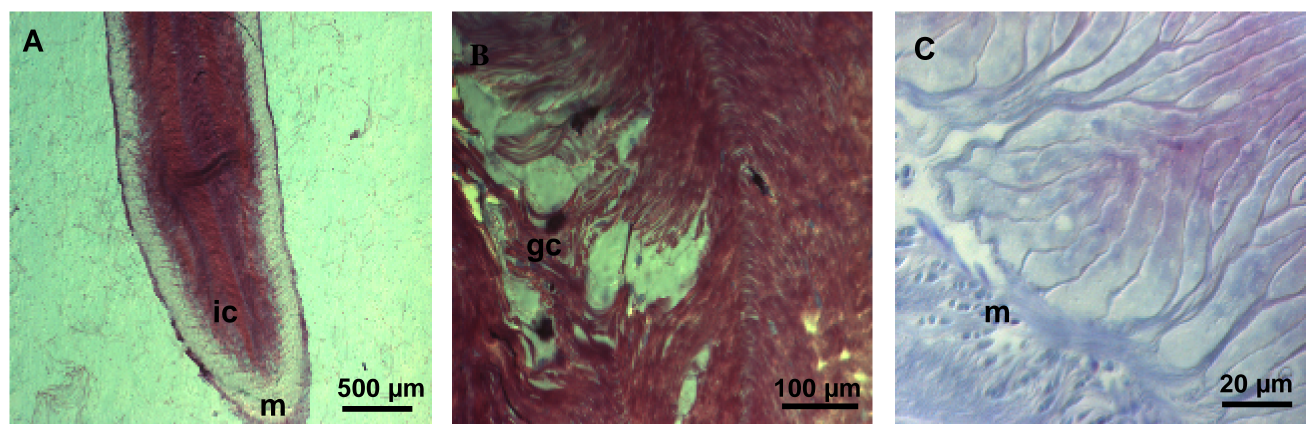


Fig. 28 Sections of Cuvierian tubules stained with Methylene blue & Azure B (**A** – 50 x magnification, **B** – 200 x magnification, **C** – 400 x magnification; **m** – mesothelium, **ic** – inner connective tissue, **g** – granular cells)

5.1.2.2 Immunostaining

The mesothelium is the tissue layer involved in adhesion. Immunostaining studies found the strongest immunoreactivity in the mesothelium, while the whole inner connective

tissue was extensively labelled. The sections were counter stained with DAPI, which was used as a nuclear stain. Controls show no immunoreactivity.

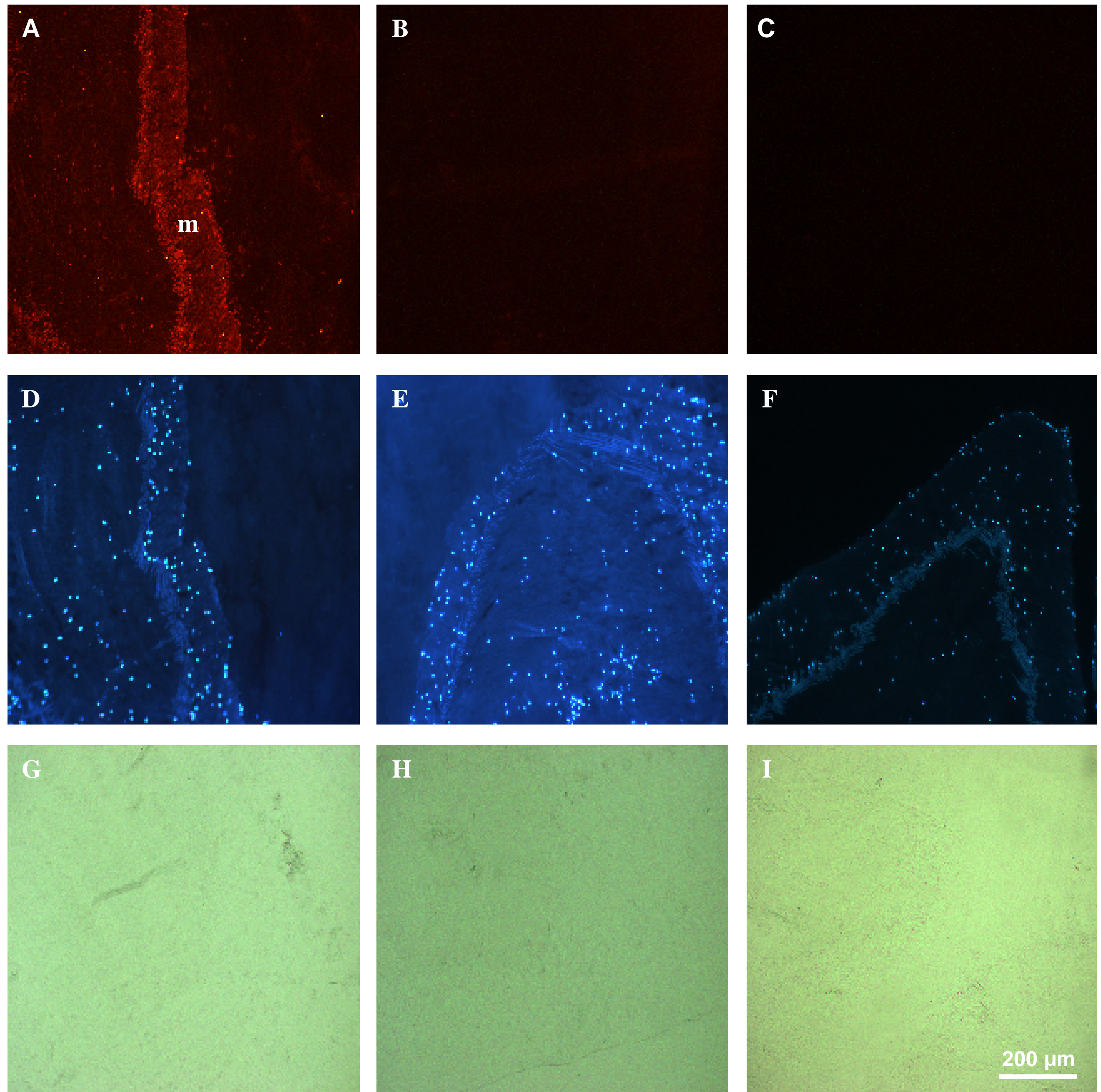


Fig. 29 Immunohistological identification of adhesive protein in sections of Cuvierian tubules. (**A** – sections of CT stained with antibody against adhesive protein 1:100 dilution, **B** – sections of CT stained with preimmiune serum 1:100, **C** – section of CT stained without primary antibody, secondary antibody for A, B, C was Cy3-conjugated goat anti-rabbit, **D, E, F** – sections stained with the DAPI, **G, H, I** – section inspected with light microscopy; **m** – mesothelium)

5.1.2.3 Immunocytochemistry (transmission electron microscopy)

Transmission electron microscopy show strong immunoreactivity in the mesothelium layer and in vacuole cells (darker area pointed by arrows on Fig. 22 A). Preimmune serum did not show any immunorectivity (Fig. 22 B).

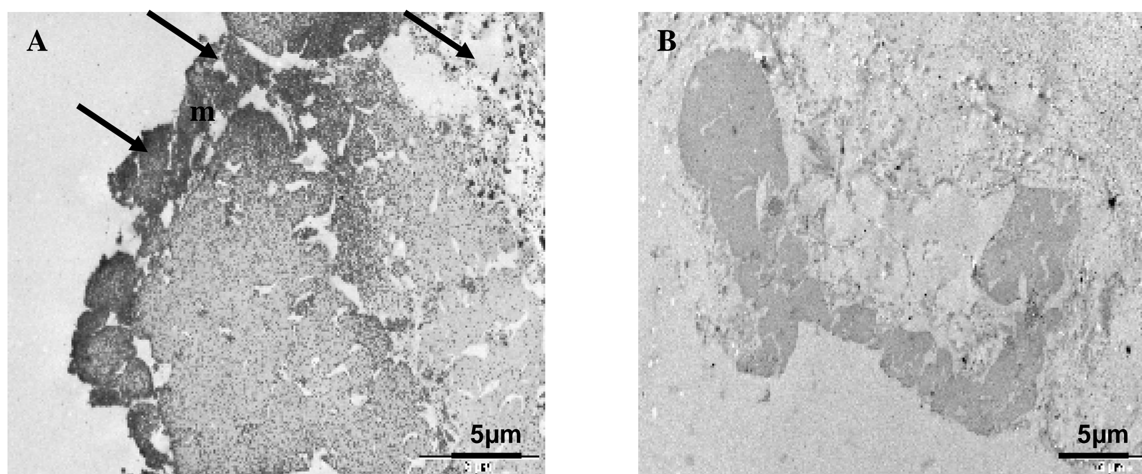


Fig. 30 Immunocytochemical location of adhesive protein in Cuvierian tubules (**A** – sections of Cuvierian tubules incubated with antibody against adhesive protein from Cuvierian tubules, **B** – sections of Cuvierian tubules incubated with preimmune serum, control;secondary antibody for A, B 1.4 – nanogold anti-rabbit IgG , **m** – mesothelium)

5.1.2.4 Measurement of adhesion

1) Adhesion of BSA

Various urea solution of BSA (1 mg/ml) did not show any adhesive properties and no adhesion was observed in the control buffers.

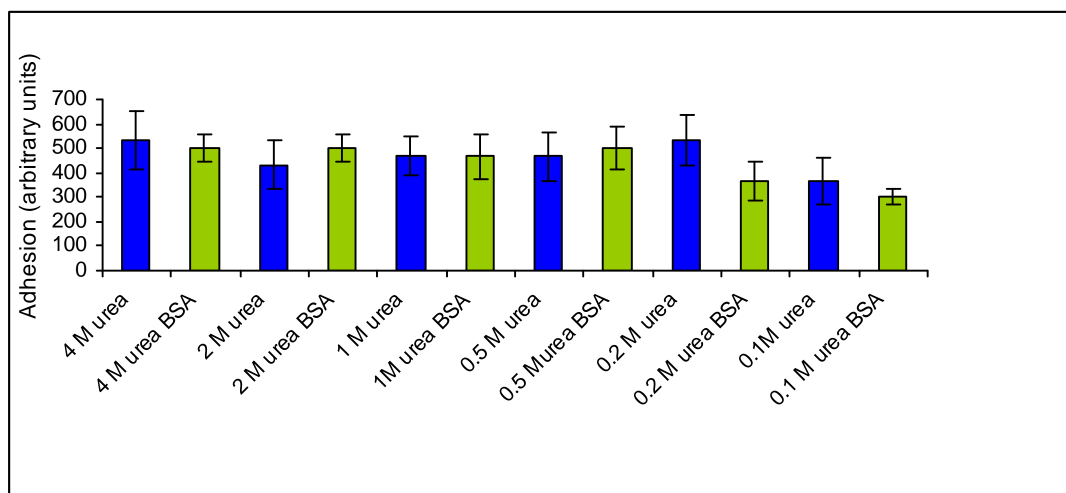


Fig. 31 Correlation between various concentration of BSA in solution of 4 M urea, 0.5 M Tris and adhesion. Results are given in arbitrary units ± SD (Standard Deviation) (n = 3).

2) Adhesion Cell-Tak

Strong adhesion of Cell-Tak was in 5% acetic acid was observed. Solution was diluted with 6 M urea, 0.75 M Tris to obtain 4 M urea, 0.5 M Tris extract. Adhesion with urea buffer was also strong but lower than adhesion with Cell-Tak in fabric formulation. Buffer showed low adhesion (Fig. 32). Results are expressed as a mean ± SD with a number of samples in parenthesis.

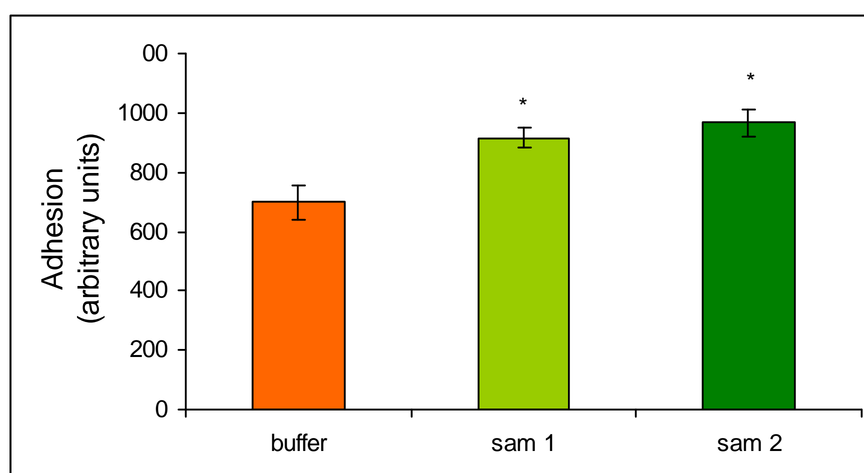


Fig. 32 Adhesion of Cell-Tak in various buffers (**buffer** – control, buffer 4 M urea, 0.5 M Tris, pH 7, **sam 1** – in 4 M urea, 0.5 M Tris pH 7.5, **sam 2** – 5 in 5% acetic acid). Results are given in arbitrary units ± SD (n = 3), *P < 0.05 (Welsch, 1977)

3) Extract of Cuvierian tubules was measured in various dilutions in urea on various surfaces

Teflon

The strongest adhesion was obtained with dilution of extract in 0.5 M urea. Higher or lower concentrations of urea resulted in lower adhesion (Fig.33). Results are expressed as a mean ± SD with a number of samples in parenthesis.

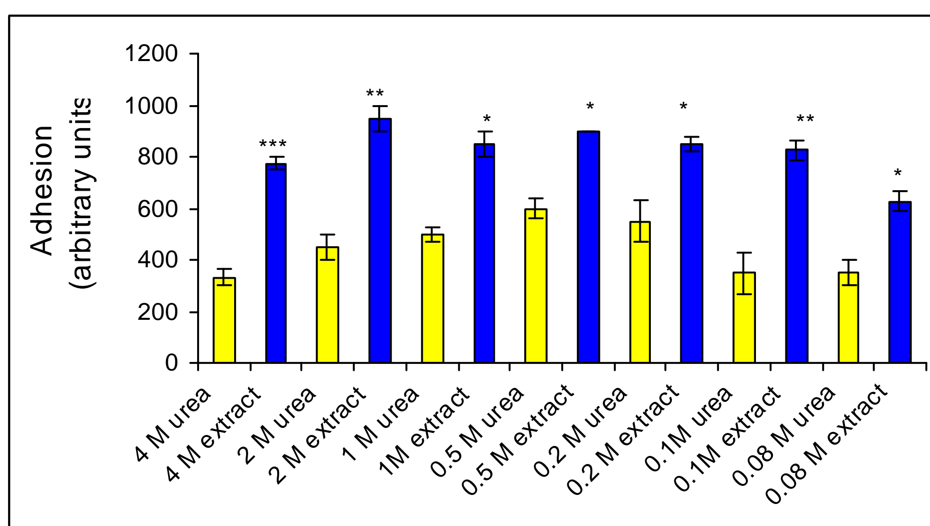


Fig. 33 Correlation between various concentration of urea and adhesion using Teflon blocks. Results are given in arbitrary units ± SD (n = 3). *P < 0.05, * * P < 0.01, *** P < 0.001 (Welsch, 1977)

Impact various concentration of protein for adhesion. Developing a standard curve using Teflon blocks

Standard curve was obtained with a logarithmic dilution of 0.5 M urea extract. Dilution of protein caused decrease of adhesion.

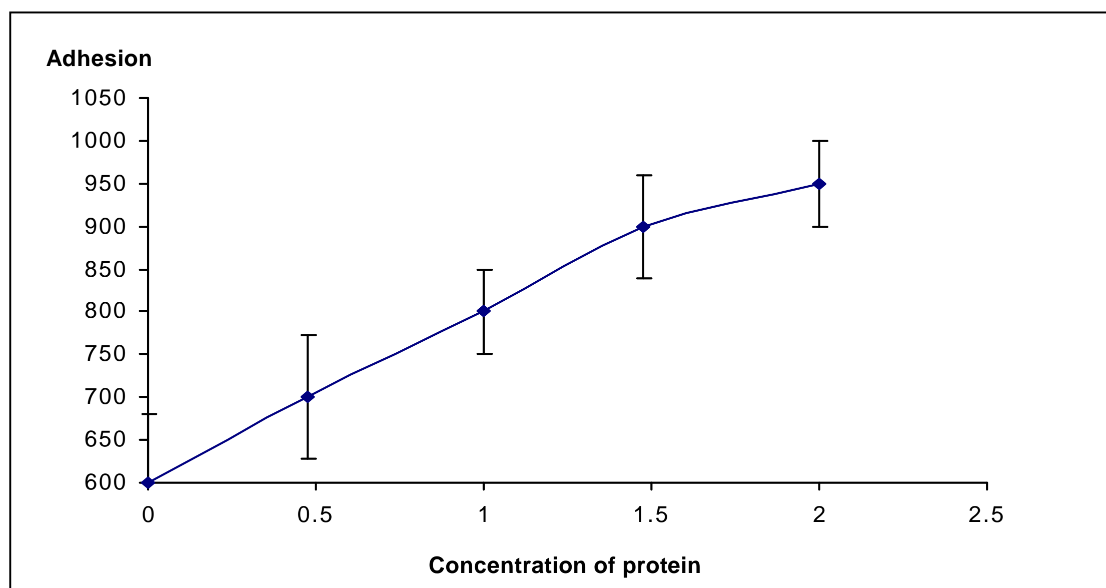


Fig. 34 Correlation between various concentration of protein in logarithmic scale and adhesion, standard curve for adhesion. Results are given in arbitrary units ± SD (n = 3).

Silicone

Various concentrations of Cuvierian tubules extract were measured using two silicone blocks. The strongest adhesion was observed in 1 M and 0.5 M urea concentration (0.25 mg/ml and 0.125 mg/ml concentration of protein). Interference from the buffer was high. Results are expressed as a mean ± SD with a number of samples in parenthesis.

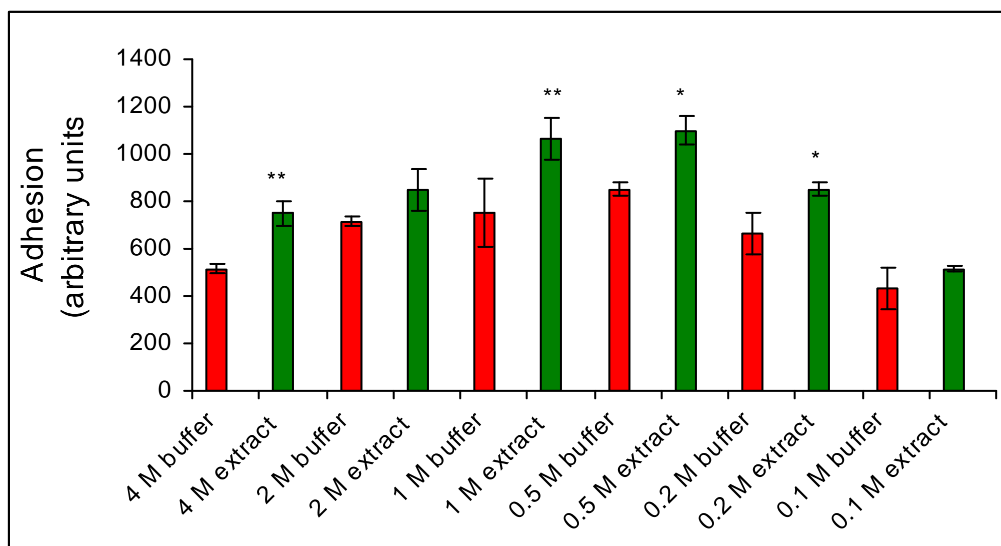


Fig. 35 Correlation between various concentration of urea extract of CT and adhesion on silicon surface. Results are given in arbitrary units ± SD (n = 3). *P < 0.05, * * P < 0.01, *** P < 0.001 (Welsch, 1977).

Gl ass

4

M urea, 0.5 M Tris Cuvierian tubules extract was diluted to several concentrations and measured using glass blocks. The strongest adhesion could be observed with 1M urea concentration of the CT (0.25 mg/ml). Interference by the extraction buffer was observed. Results are expressed as a mean ± SD with a number of samples in parenthesis.

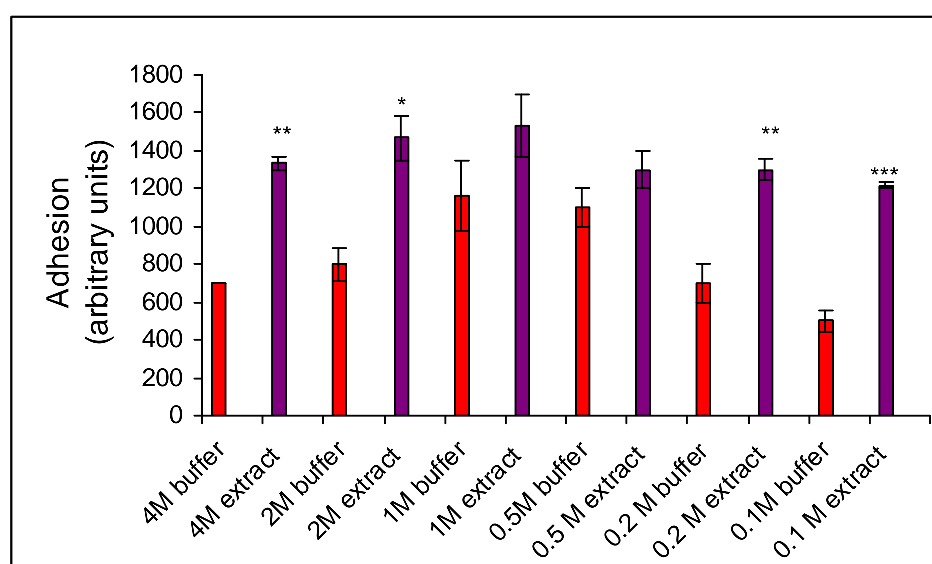


Fig. 36 Correlation between various concentration of urea extract of CT and adhesion on glass surface. Results are given in arbitrary units ± SD (n = 3). *P < 0.05, * * P < 0.01, *** P < 0.001 (Welsch, 1977)

Iron

Extract of Cuvierian tubules was diluted to several dilutions and measured using two iron blocks. Strong interference of the extraction buffer could be observed at almost all concentrations. The strongest adhesion could be observed with 1 M urea extract (0.25 mg/ml). Results are expressed as a mean ± SD with a number of samples in parenthesis.

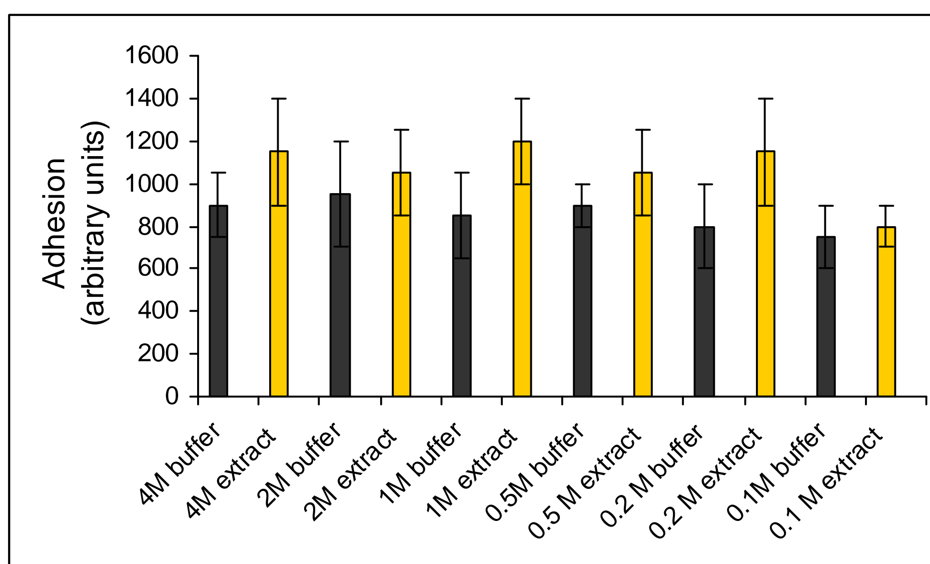


Fig. 37 Correlation between various concentration of urea extract of CT and adhesion on glass surface. Results are given in arbitrary units ± SD (n = 3). *P < 0.05, * * P < 0.01, *** P < 0.001 (Welsch, 1977)

Gelatine

Various concentrations of CT extract were measured using blocks cover with gelatine. Measurement was interrupted by the buffer which reacted with the gelatine. The highest adhesion of the extract could be observed with 2 M urea extract (0.5 mg/ml). Results are expressed as a mean ± SD with a number of samples in parenthesis.

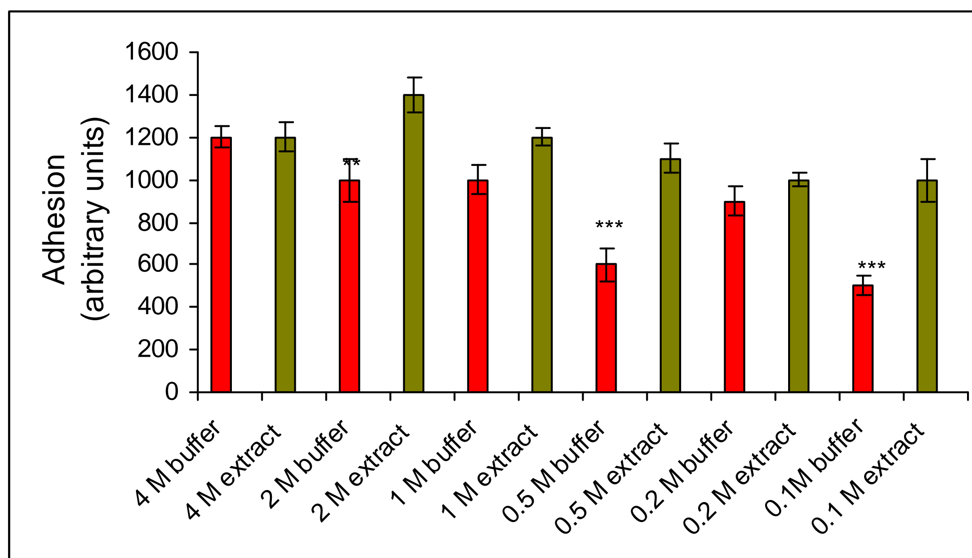


Fig. 38 Correlation between various concentration of urea extract of CT and adhesion on gelatine surface. Results are given in arbitrary units ± SD (n = 3). *P < 0.05, * * P < 0.01, *** P < 0.001 (Welsch, 1977)

4) Impact of metal cations and EDTA on adhesion

The tensile strength in Cuvierian tubules was measured in the presence of K^{+} (100 mM and 50 mM concentration), Ca^{2+} (5 mM), Zn^{2+} (5 mM),EDTA (10 mM) and Cd^{2+} (5 mM). Measurement showed that adhesion decreased by potassium and EDTA and decrease by zinc and cadmium. Results are expressed as a mean ± SD with a number of samples in parenthesis.

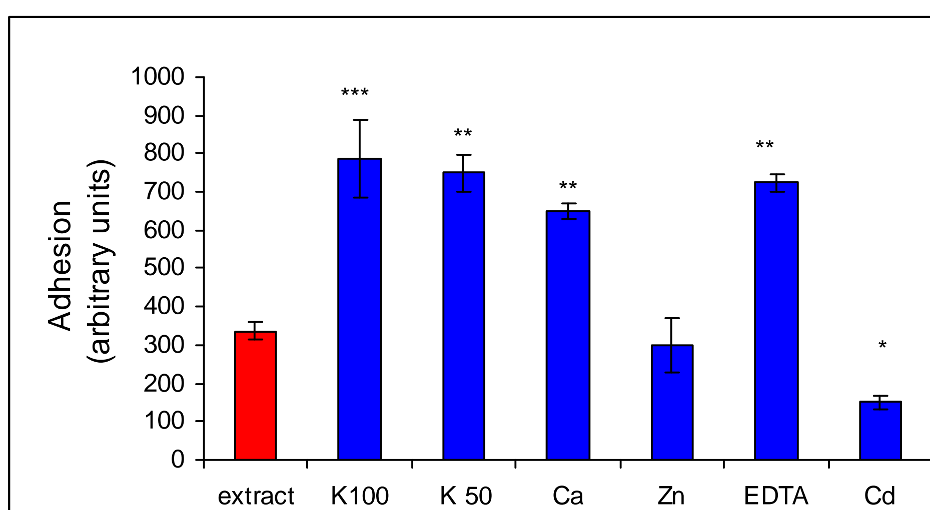


Fig. 39 Impact various metal cations and EDTA on adhesion in 0.5 M urea extract. Results are given in arbitrary units ± SD (n = 3). *P < 0.05, * * P < 0.01, *** P < 0.001 (Welsch, 1977)

5) Measurement of adhesion of fractions from gel filtration column

Fractions obtained during isolation of adhesive protein by gel filtration were measured for adhesion. The strongest adhesion was obtained in fraction 7 where the concentration of adhesive protein was the highest. Electrophoresis gel protein content in each fraction is described in paragraph 5.1.1.3.3. Results are expressed as a mean ± SD with a number of samples in parenthesis.

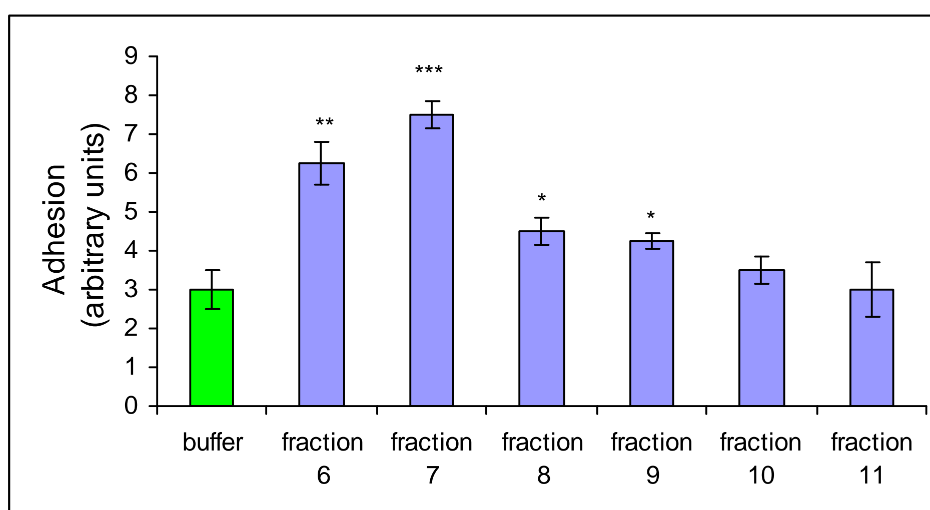


Fig. 40 Diagram presenting adhesion of fractions from gel filtration. Results are given in arbitrary units ± SD (n = 3). *P < 0.05, * * P < 0.01, *** P < 0.001 (Welsch, 1977)

6) Measurement of adhesion of the gel filtrations fractions with the metal cations and EDTA

Each fraction eluted from gel filtration containing adhesive protein was tested for impact of K^{+}, Ca^{2+}, Mg^{2+} and EDTA. Cations had impact on adhesion in each fraction. Potassium increase adhesion in every fraction.

Presence of calcium increase adhesion in fractions were adhesive protein was present in higher concentration.

Presence of magnesium had no significance difference on adhesion. EDTA increased adhesion in fractions with high concentration of adhesive protein.

7) Adhesion of the extract neutralised by

Antibody

Neutralisation of antibody was carried out as described in Material and Methods. Adhesion of neutralised extract decreased in comparison to the non neutralised extract. It can be concluded that antibody bound to the adhesive protein and inhibits the adhesion. Results are expressed as a mean ± SD with a number of samples in parenthesis.

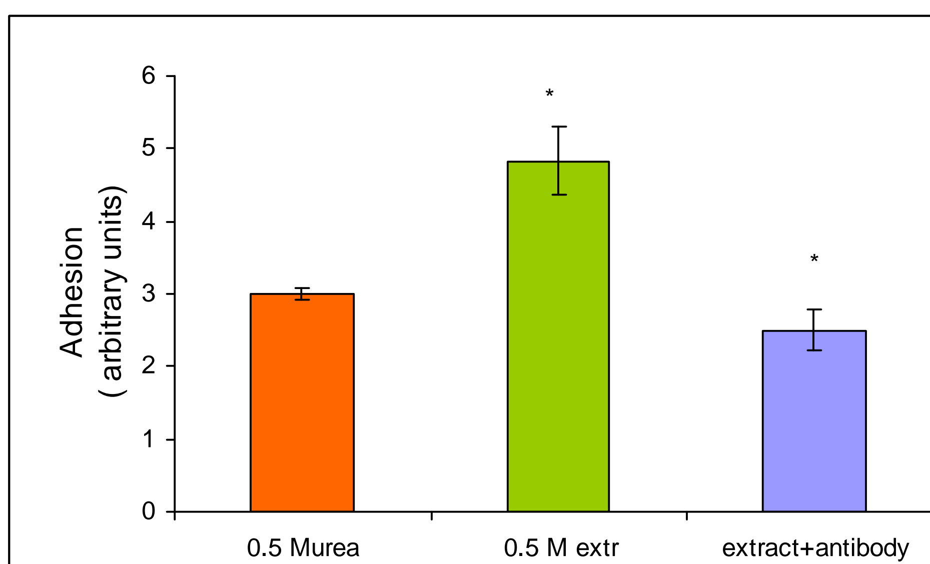


Fig. 41 Adhesion of extract neutralised by antibody. Results are given in arbitrary units ± SD (n = 3). *P < 0.05, * * P < 0.01, *** P < 0.001 (Welsch, 1977)

Trypsin

Neutralisation by trypsin was carried out like described in Materials and methods**.** Adhesion of the neutralised extract was used as a positive control. Extracts incubated with trypsin show inhibition of the adhesion. Results are expressed as a mean ± SD with a number of samples in parenthesis.

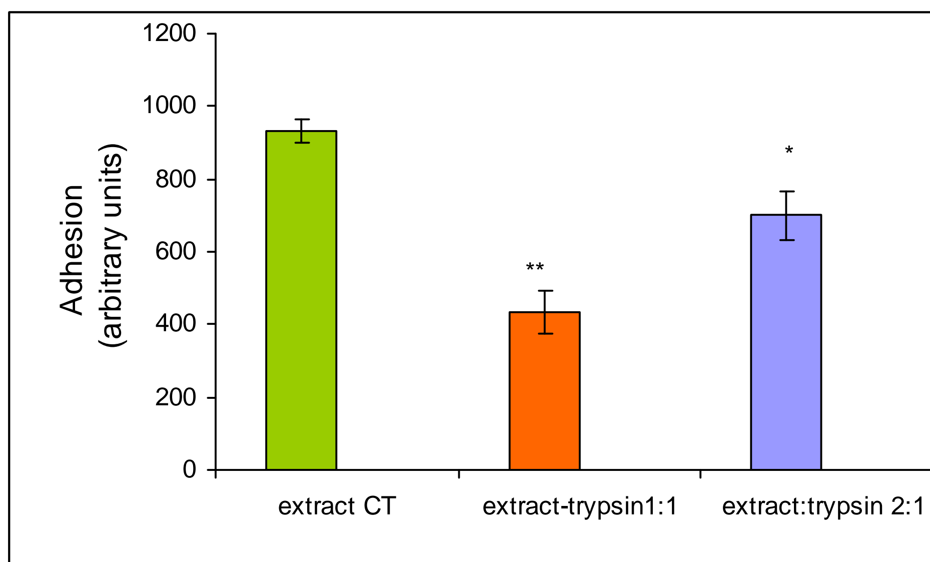


Fig. 42 Adhesion of extract neutralised by trypsin. Results are given in arbitrary units ± SD (n = 3). *P < 0.05, * * P < 0.01, *** P < 0.001 (Welsch, 1977)

8) Adhesion of mussels extract

Extract from the mussels *Mytilus galloprovincialis* in 4 M urea, 0.5 M Tris pH 7.5 were measured in the same condition as that of the extract from the Cuvierian tubules. Various dilutions were made to compare the adhesion dependant of the urea and protein concentrations. The highest adhesion was obtained with 4 M urea extract (Fig. 30). Results are expressed as a mean ± SD with a number of samples in parenthesis.

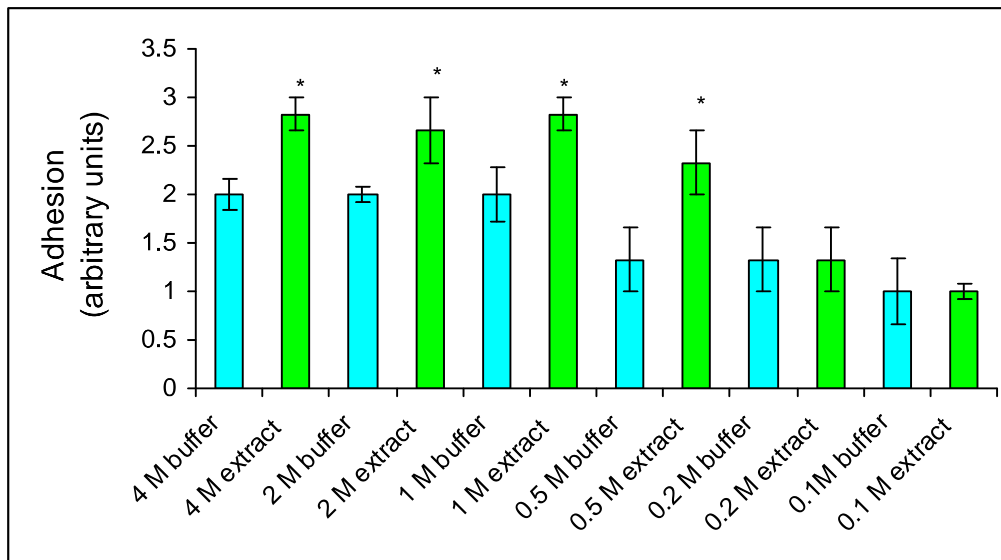


Fig. 43 Correlation between various concentration of mussels extract and adhesion. Results are given in arbitrary units ± SD (n = 3). *P < 0.05, * * P < 0.01, *** P < 0.001 (Welsch, 1977)

9) Adhesion of silk fibroin

Silk fibroin showed strong adhesion, similar to the Cuvierian tubules extract.

Results are expressed as a mean ± SD with a number of samples in parenthesis.

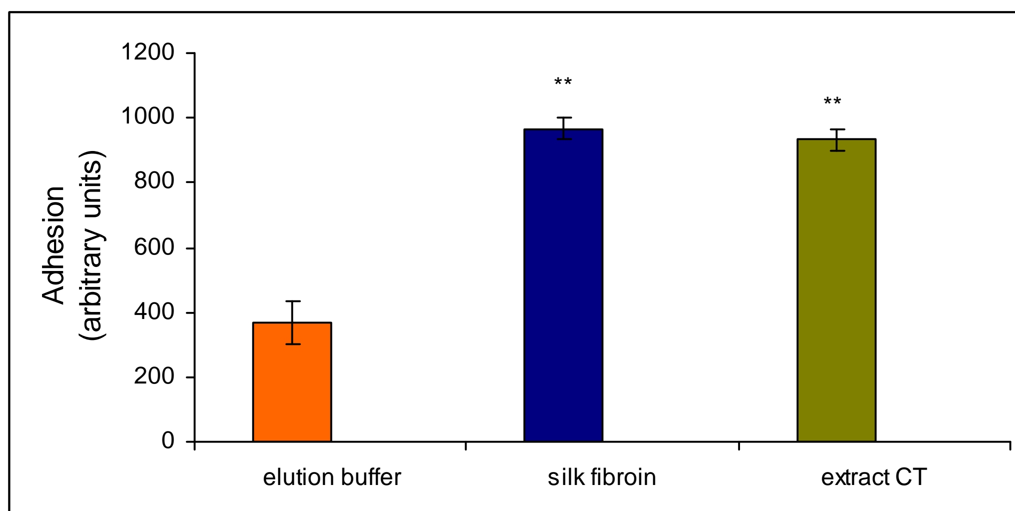


Fig. 44 Adhesion of elution buffer of silk fibroin, silk fibroin and Cuvierian tubules extract. Results are given in arbitrary units ± SD (n = 3). *P < 0.05, * * P < 0.01, *** P < 0.001 (Welsch, 1977)

5.1.2.4 Labelling adhesive protein in mussels threads using antibody against adhesive protein from Cuvierian tubules

Mussels *Mytilus galloprovincialis* were allowed to adhere to the surface of slides. When it was observed that mussels attach to the surface, the animals were removed. Mussel byssus stained on slides with antibody against adhesive protein showed immunoreactivity around the threads where the adhesive protein is probably localised. Controls showed no immunoreactivity.

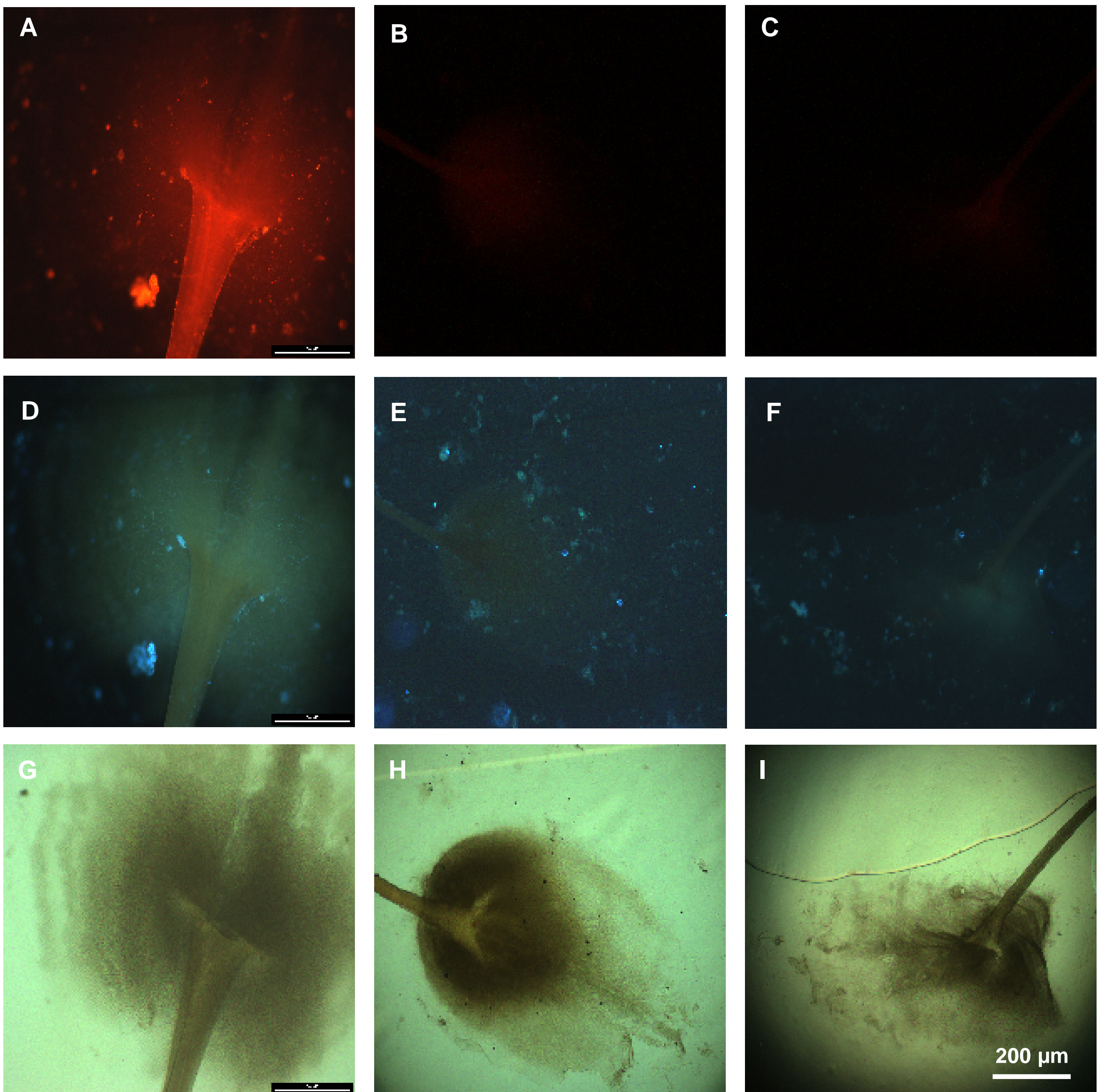


Fig. 45 Labelling adhesive protein in the byssus of mussels (**A** – byssus incubated with antibody against adhesive protein from CT (1:100), **B** – byssus incubated with premium serum (1:100), control **C** – byssus incubated in blocking solution without antibody (control), **D, E, F** – byssus stained with DAPI, **G, H, I** – byssus inspected by light microscopy)

5.2 STARFISH *MARTHASTERIAS GLACIALIS*

5.2.1 Isolation of adhesive protein from the tube feet and footprint of starfish

5.2.1.1 Protein extraction

Adhesive material was obtained by cutting the tube feet from the specimen and by collection scraped from glass to which footprint were attached. Solubilisation of this material proved difficult particularly in the choice of a good buffer for homogenization, which solubilised the material, but did not cause complications for subsequent analysis. Urea and SDS buffers have proved the most useful in digesting the tissue but with the resulting extract still being suitable for gel electrophoresis. Guanidine buffer was effective at solubilising the material, but there were problems with the subsequent precipitation of the extract during dialysis. Fig 46 is the visualisation of a SDS gel with homogenised tube feet in various buffers. Homogenisation with PBS (Fig. 46, lane 3), showed a lack of bands indicating that this buffer didn't solubilise the tissue. Tissue homogenised using urea buffer (Fig. 46, lane 2) and SDS buffer (Fig. 46, lane 1) showed good bands confirming that these buffers are suitable to solubilised the material.

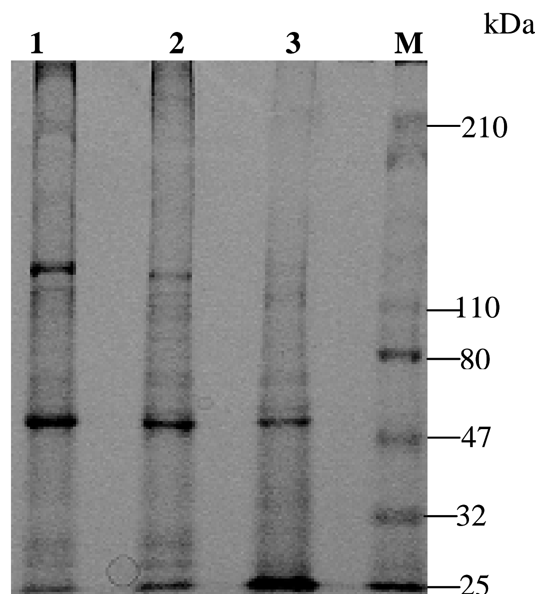


Fig. 46 Various buffers used for homogenisation of the tube feet from *Marthasterias glacialis* present on 10 % SDS gel, stained with Coomassie Blue (**1** – 5% SDS buffer, **2** – 2 M urea buffer, **3** – PBS, **M** – marker).

5.2.1.2 Purification of extract for SDS-PAGE

The SDS-PAGE assay was used to approximate the protein sizes and their concentration. Crude homogenized extract merely appeared as a smear on the SDS-PAGE making it impossible to distinguish any bands. To improve the analysis the extract was subjected to clean up steps.

Dialysis was the first step in purification procedure. Several buffers were tested for dialysis (see Material and Methods). During dialysis, the protein sometimes precipitated, especially with extraction buffers containing high concentrations of guanidine or urea, making it difficult to analyse the sample. As dialysis diluted the sample, a further concentration step was necessary.

Spin columns were used for concentration of the sample though these did not always give satisfactory results. Comparing the complete extract to that remaining after use of the spin column, showed that a lot of material had been retained in the spin column. This may be due to several reasons:

i) the spin column was over loaded

ii) the sample blocked the column

iii) the sample contained large proteins which couldn't pass through the column.

Precipitations methods were also used to clean up and concentrate the sample. Kits used for purifications as described in Material and methods helped to obtain highly purified pellets of the protein, though these sometimes were difficult to dissolve in the sample loading buffer.

Organic solvents like ethanol and acetone were very useful to obtained purified protein but sometimes solubilisation of the purified protein pellet caused difficulties.

5.2.1.3 Analysis tube feet and footprint by electrophoresis gel after purification

Before electrophoresis analysis, standard Bradford protein assays were carried out on the extracts of tube feet to assess their protein content. The concentration of protein in the sample was about 6 mg/ml. Some of the extraction buffers reacted with the Bradford Reagent, requiring either a change of buffer, or by dialysis prior to the assay.

The concentration of protein in the footprint was assessed by the 2-D Quant Kit compared to the standard curve. The concentration of protein was much lower in the footprint than in the tube feet (50 µg/ml as opposed to 6 mg/ml).

SDS-PAGE analysis was repeated after prior purification of the crude extract. The adhesive protein could be identified in the lane of the tube feet. The tube feet extract posessed large amounts of proteins with various sizes. Molecular weight of the highest concentration protein were approximately 47, 50, 60, 100, 110, 150 kDa.

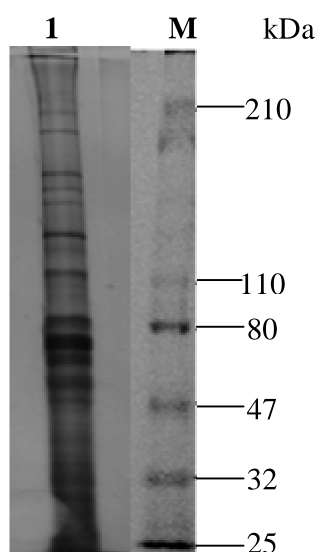


Fig. 47 Tube feet from the starfish *Marthasteria glacialis* homogenised using 2 M urea buffer and purifed by dialysis and Clean up Kit analysed by 10% SDS gel, stained with silver stain (**M** – marker, **1** – in 2 M urea buffer pH 8.8).

To localise the adhesive protein amongst these various tube foot proteins, the footprint extract was examined. Footprints which developed after adhering starfish to the surface are made up of adhesive protein. After washing with solvent to removed any impurity, only proteins involved with adhesion remained behind though the concentration of these proteins was very low. Several dyes were tested to detect proteins in the footprint; sensitivity of the dye was main reason for selection of GelCode Blue Reagent, Glycoprotein stain, silver stain, Sypro Ruby and Imperial Stain.

GelCode Blue Stain Reagent was not sufficiently sensitive for the footprint, though it worked well with the main bands in the tube feet extract. Only a smear could be

observed in the lane of the footprint, while in tube feet a band around 50 kDa was clear visible, though the remaining bands were difficult to visualise.

Glycoprotein staining didn't detect any glycoprotein in the footprint; in the tube foot extract there was only a smear and no bands were visible.

After staining with silver stain numerous protein bands were visible in the tube foot extract. The staining of the footprint extract was smeary but weak bands were seen. Silver staining is more sensitive than Coomassie staining and is more useful when working with footprint material but still the results were still not satisfactory.

The fluorescent dye, Sypro Ruby, was one of the most sensitive dyes was tested with the footprint and tube feet extract. In the lane of the tube feet extract some bands were observed around 30-80 kDa. In the footprint lane it was difficult to recognize any bands, as only a smear appeared. This dye also appeared to be insufficiently sensitive to detect the footprint proteins. Extract of footprints and tube feet were stained with Imperial stain, which is based on Coomassie dye but has additional components, which makes this stain more sensitive. In the footprint lane one main band was observed around 50 kDa. Summary of the tests with stains are present in Table 3.

Table 3 Summary of the test with various stains.

	GelCode Blue Stain Reagent	Glycoprotein stain	Silver stain	Sypro ruby stain	Imperial stain
Tube Feet	Stain band around 50 kDa strong, many weak bands	Only smear observed	Several bands could be observed, stain very well	Some band could be observed	Many bands could be observed
Footprint	Not enough sensitive	Lack any signal	Only smear could be observed	Only smear could be observed	Band at 50 kDa could be recognised

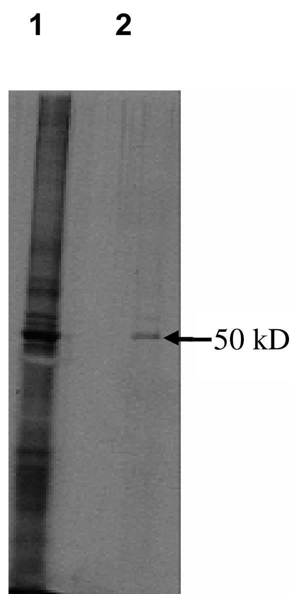


Fig. 48 Analysis of tube feet and footprint from *Marthasterias glacialis*. Samples were separated on 10% SDS-PAGE gel, loading 30 µl (**1 –** protein from footprint in 2 M urea, 0.1 M DTT, 63 mM Tris-HCl, pH 8.0 buffer, **2 –** total protein extract from tube feet in 2 M urea, 0.1 M DTT, 63 mM Tris-HCl, pH 8.0 buffer)

5.2.1.4 Purification of the adhesive protein using HPLC

To obtained pure protein necessary for further analysis, a method of digestion was used. Extract was loaded on several gels and stained with Imperial Blue Stain. Bands of 50 kDa were cut from the gel and digested. The presence of the protein and its purity were checked on HPLC and in parallel, freshly collected footprints were tested. Samples were injected onto two columns: size exclusion column (BioSep-SEC-S4000) and reverse column (Synergi MAX-RP C12 Reverse).The Size exclusion column (SEC) identified the molecular mass of the protein in the sample. BSA was run as a standard (molecular mass 66 kDa) and the retention time of this protein was found to be around 18 minutes. Samples of fresh footprint were run in the same condition as the standard, and the molecular mass of the protein was estimated to be around 50 kDa (retention time 25 minutes).

Samples were run on the reverse column, in different condition in organic solvents to show purity of the sample. This confirmed that the peak obtained in chromatogram from the SEC column belongs to the sample and is not a contamination from solvents or column.

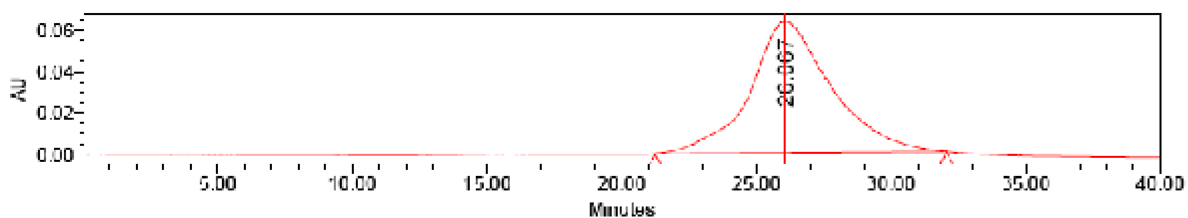


Fig. 49 Chromatogram of sample of the footprint collected by wooden stick, dissolved in CH_3CN and injected on HPLC (column BioSep-SEC- S4000).Conditions: isocratic 100% 40 mM NaH_2PO_4 , pH 6.8, retention time 26.06 (A: 0.6).

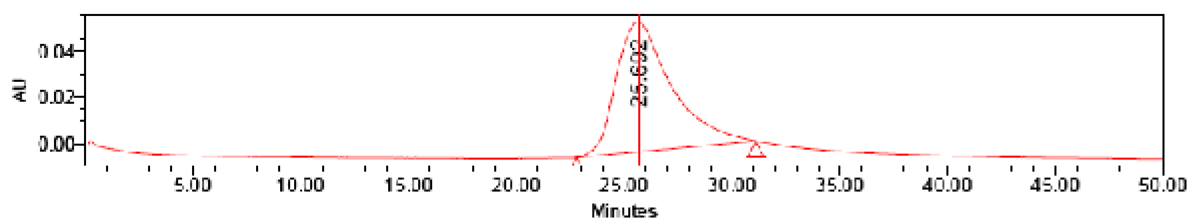


Fig. 50 Sample of the footprint scraped from glass plates using a razor was dissolved in MeOH and injected to an HPLC (column Synergi MAX-RP C12 Reverse). Conditions: gradient 90% H_20, 10% MeOH, time: 50 min. To 100% MeOH Retention time: 25.692 (A:0.5).

The peak observed in the chromatogram at 25 min had high absorption (0.5). HPLC caused lot of problems with analysing the protein. It was very difficult to find appropriate buffer for injection as buffer which could solubilise the sample could not be injected onto some of the columns due to incompatible chemistries. Low concentration of the sample caused problems for detection with HPLC because of sensivity issues if the concentration of sample is low.

5.2.2 Characterisation of adhesive protein in the tube feet and footprint of starfish *Marhasterias glacialis*

5.2.2.1 Histology

Footprint

To further characterise the footprint, preparations were made on glass slides for analysis using the light microscope. Footprints were stained using 1) Alcian blue; 2) Haematoxylin & Oil Red O; 3) Ponceau; 4) Alcian blue/PAS staining; 5) Alcian blue/Ponceau/Acid fuschine; 6) Coomassie Blue 7) Toluidyne blue

The footprint was stained strongly using alcian blue, indicating the presence of substantial amounts of acid-mucopolysaccharides. Ponceau and Acid fuchsine pink also stained certain components within the footprint, which may indicate the proteinaceous adhesive secretion.

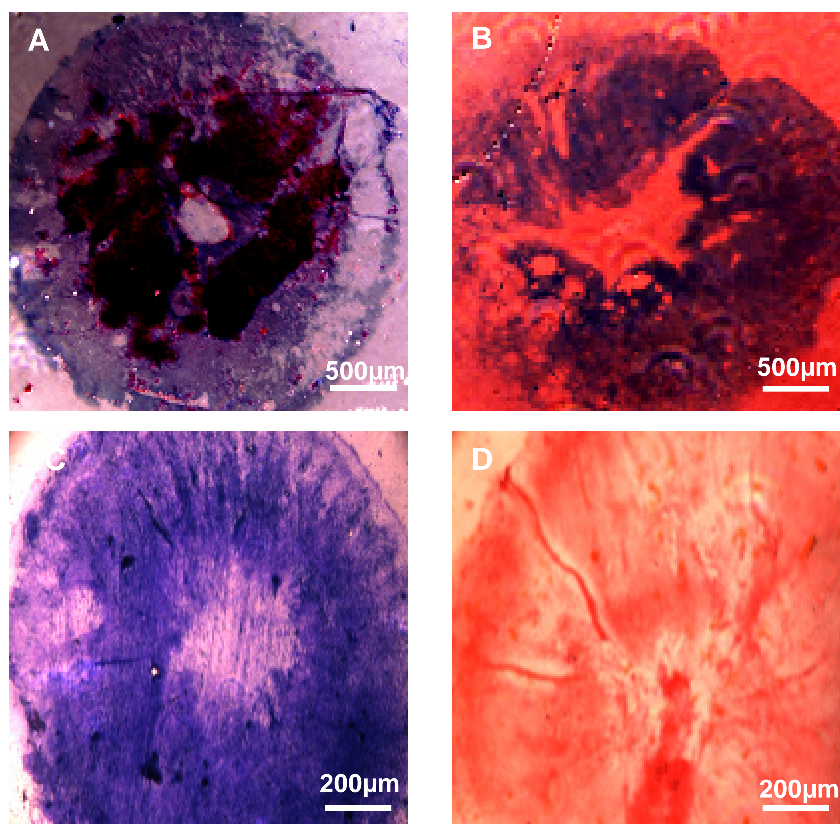


Fig. 51 Histology of the footprint (**A** – Poncau/Acid fuschine 50 x magnification, **B** – Alcian blue 50 x magnification, **C** – Coomassie Blue 100 x magnification , **D** – H&E 100 x magnification)

Tube feet

The histological structure of the tube feet is remarkably constant for all echinoderm species. Their tissue stratification consists of four layers: an inner myomesothelium surrounding the ambularcral lumen, a connective tissue layer, a nerve plexus and an outer epidermis covered externally by a cuticle (Flammang 1996).

Tube feet of *Marthasterias glacialis* consist of a reinforced disc-ending (Santos, 2005), which has similar external morphology to simple disc-ending feet. In the sections it was possible to observe radial lamellae of connective tissue surrounded by adhesive epidermis.

The tube foot stained strongly with Haematoxylin & Eosin (Fig. 38 1a, 1b). It possible to observed the adhesive epidermis staining red and nuclei staining blue-purple. In tube feet stained with the Casson's Trichrome (Fig.38 2a, 2b) the epidermis stained pink and the connective tissue stained blue.

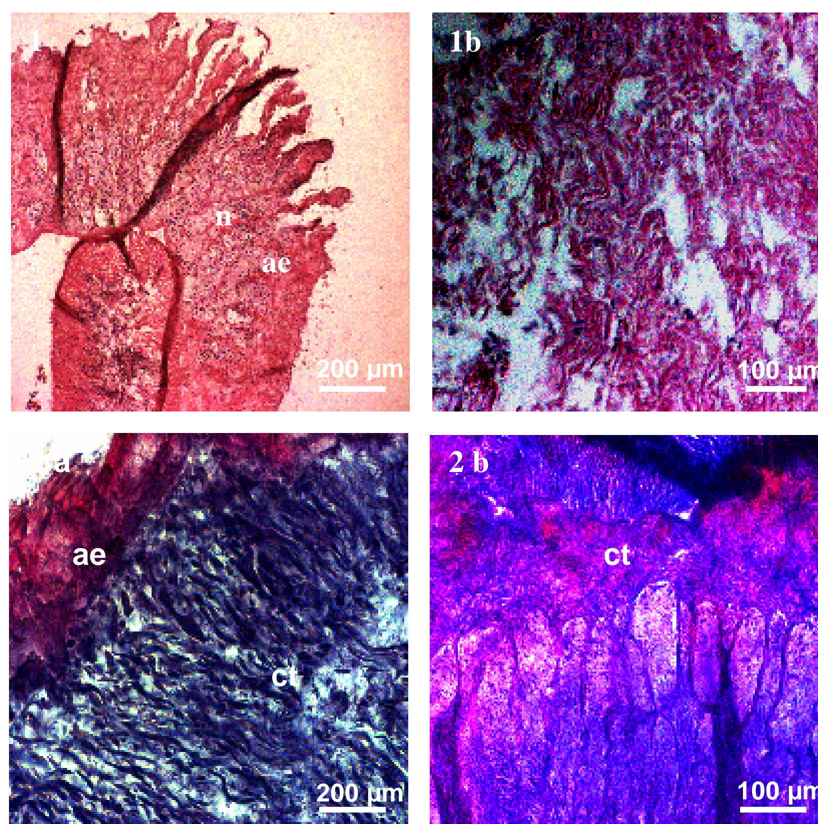


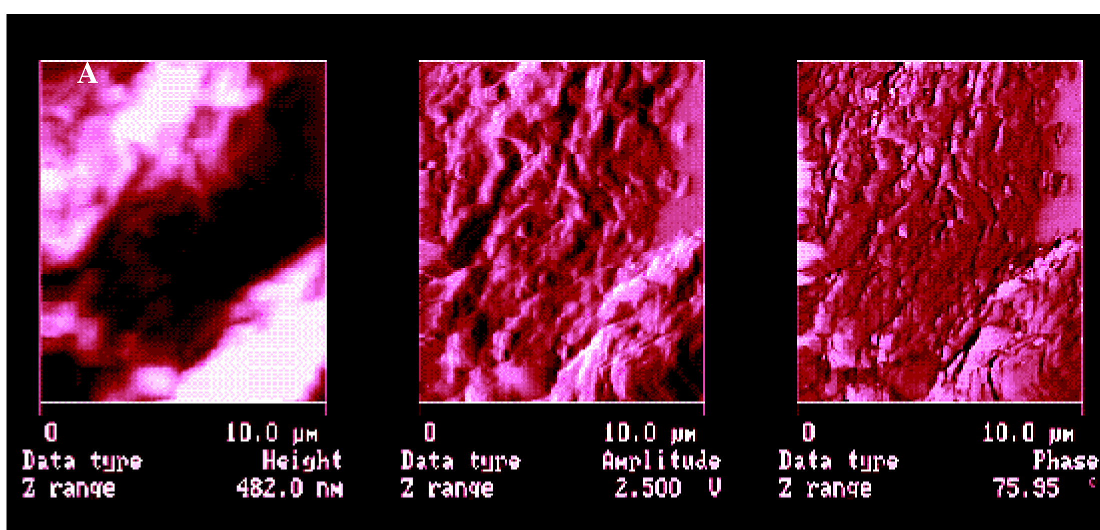
Fig. 52 Histology of the tube feet (**1a** – H&E 100 x magnification, **1b** – H&E 200 x magnification, **2a** – Casson's Trichrome 100 x magnification, **2b** – Casson's Trichrome 200x magnification, **ae** – adhesive epidermis, **n** – nuclei, **ct** – connective tissue)

5.2.2.2 AFM

Many attempts were made to investigate the nature of the starfish footprint using AFM under various conditions. A sample of the footprint was scanned in wet tapping mode in water using the AFM Bioscope (Veeco). AFM images showed the topographical structure of the footprint, depending on the scanned area. AFM was also used to try and measure adhesion over the area of the footprint, although because of the stickiness of the material, this can result in the probe damaging the footprint. If prints are fixed in formaldehyde, this stickiness is lost. Structure of the footprint was analysed by height, amplitude and phase images (Fig. 53 A).

Height image present a topographical image of the footprint. The height and shape of the footprint on the surface is shown by this image. The Amplitude measured the error/displacement on the tip, during scan of the footprint.

A phase image, is texture of the surface, it measures the roughness and some extent "stickiness" of a footprint by the amount of drag on the tip. Phase image showed details of the footprint, in 3D image (Fig 53 B) is possible to observed complicated structure of the footprint.



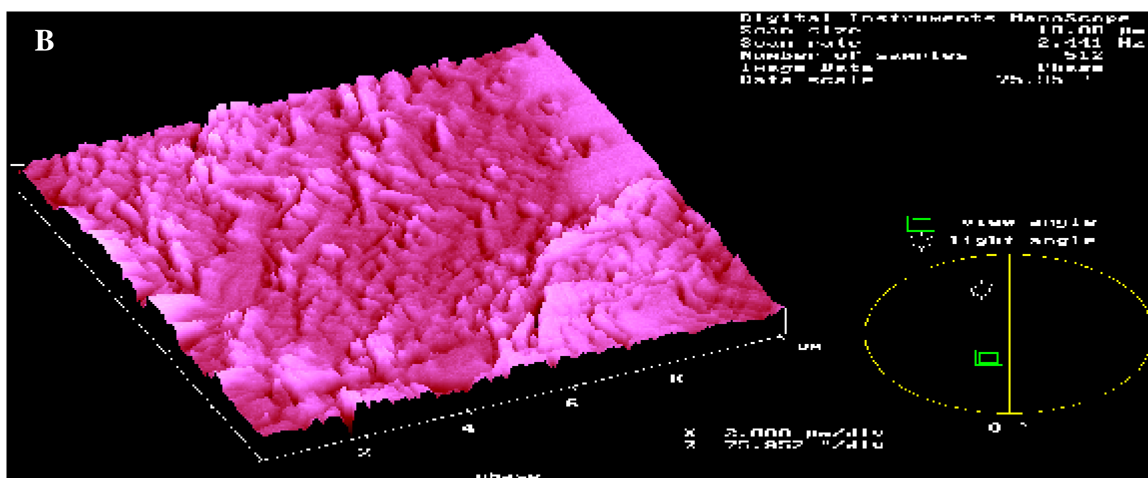


Fig. 53 Images of footprint scanned by AFM in tapping mode in wet condition (**A –** image of footprint in height, amplitude and phase image, 10 µm, **B –** image of footprint in 3D image phase, scan 10µm)

Force calibration showed adhesion of the footprint which is relatively high.

The force curve plot is read as a cycle of the tip's motion toward the away from the sample surface. See Fig. 54

Segement 1-2 tip moving toward the sample.

Segment 2-3 shows the cantaliver bending up as the tip is pushed into the sample surface

Segment 3-4 shows the cantaliver moving down as the tip is pulled away from the surface

Segment 4-5 shows the point where the tip detaches from the sample surface

Segment 5-6 shows the cantaliver position has returned as the tip is no longer influenced by the sample

Force calculation: $F = k \times z$

k – spring constant of the cantilever (here: 0,60 N/m)

$z = \Delta Z \times S$ (ΔZ – distance between two positions on plot (here: 72,24 V); S – Sensivity (here: 86.25 nm/V)

F = 3738,42 nN

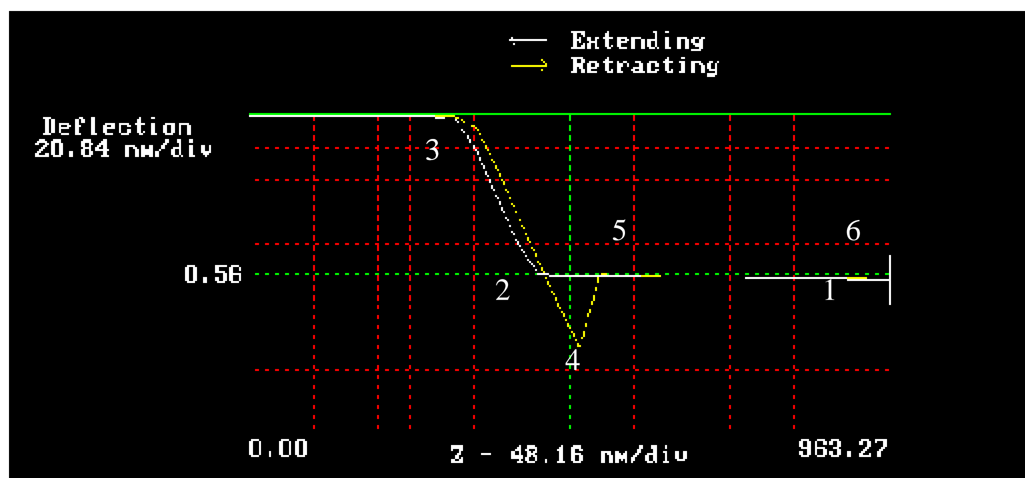


Fig. 54 Force calibration plot in Contact Mode measure footprint (scan size 10 µm)

5.2.2.3 Wyko NT 1100 (optical profiler)

Wyko (owned by VEECO Cambridge) provides non-contact imaging at high speed with a large field of view. Images of the footprint scan by Wyko in dry conditions have helped to determine the structure of the footprint. The footprints were on average 2 mm in diameter and the surface. Back surface of the footprint o is very complicated, displaying a network of material, with varying composition. Various colours on images showed that the footprint is not flat (Fig. 55 A). Surface of the footprint is some areas is quite high (red colours on Fig. 55 B) but there are areas in the footprint where secretion is not present (blue colours on Fig. 55 B).

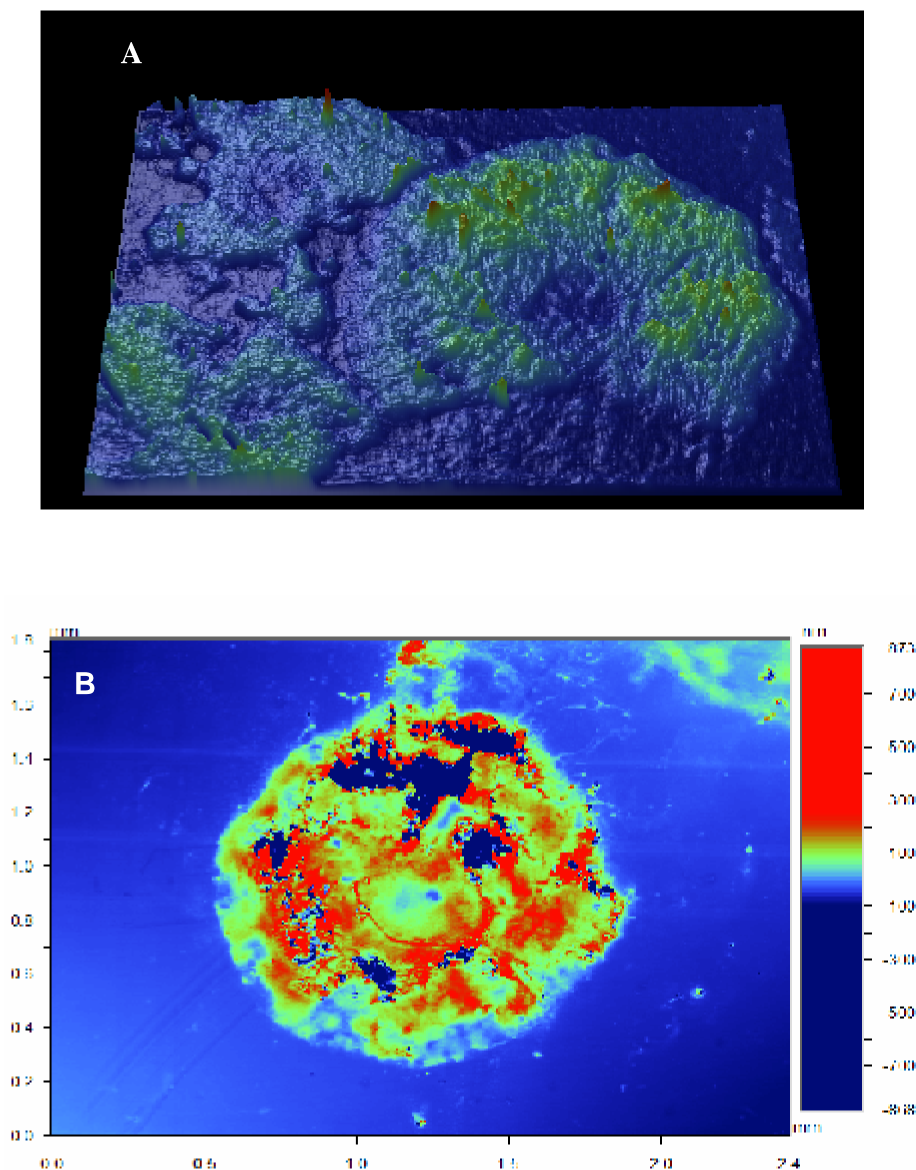


Fig. 55 Images of footprint scanned by Wyko NT1100

(**A** – 3D image, **B** – 2.5 x magnification, profile of the footprint)

5.2.2.4 Characterisation of the adhesive protein in starfish extract using antibody against adhesive protein from Cuvierian tubules

Extract of starfish tube feet was run on a gel and transferred to the membrane which was incubated with antibody against adhesive protein from Cuvierian tubules. Two very strong band and other small bands were seen (Fig. 56).

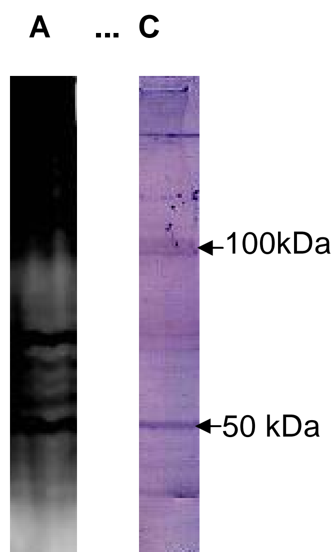


Fig. 56 Labelling adhesive protein in starfish extract using antibody against adhesive protein from Cuvierian tubules *Holothuria forskali* (**A** – extract of tube feet from starfish loaded 12% SDS PAGE gel, **B** – Western blot of the tube feet extract incubated with antibody against adhesive protein from Cuvierian tubules and developed with antibody anti - rabbit IgG alkaline phosphatase).

Immunostaining had been done in order to localise adhesive protein in sections of tube feet (Fig. 57). Strong immunoreactivity was observed in adhesive epidermis. Controls showed no immunoreactivity.

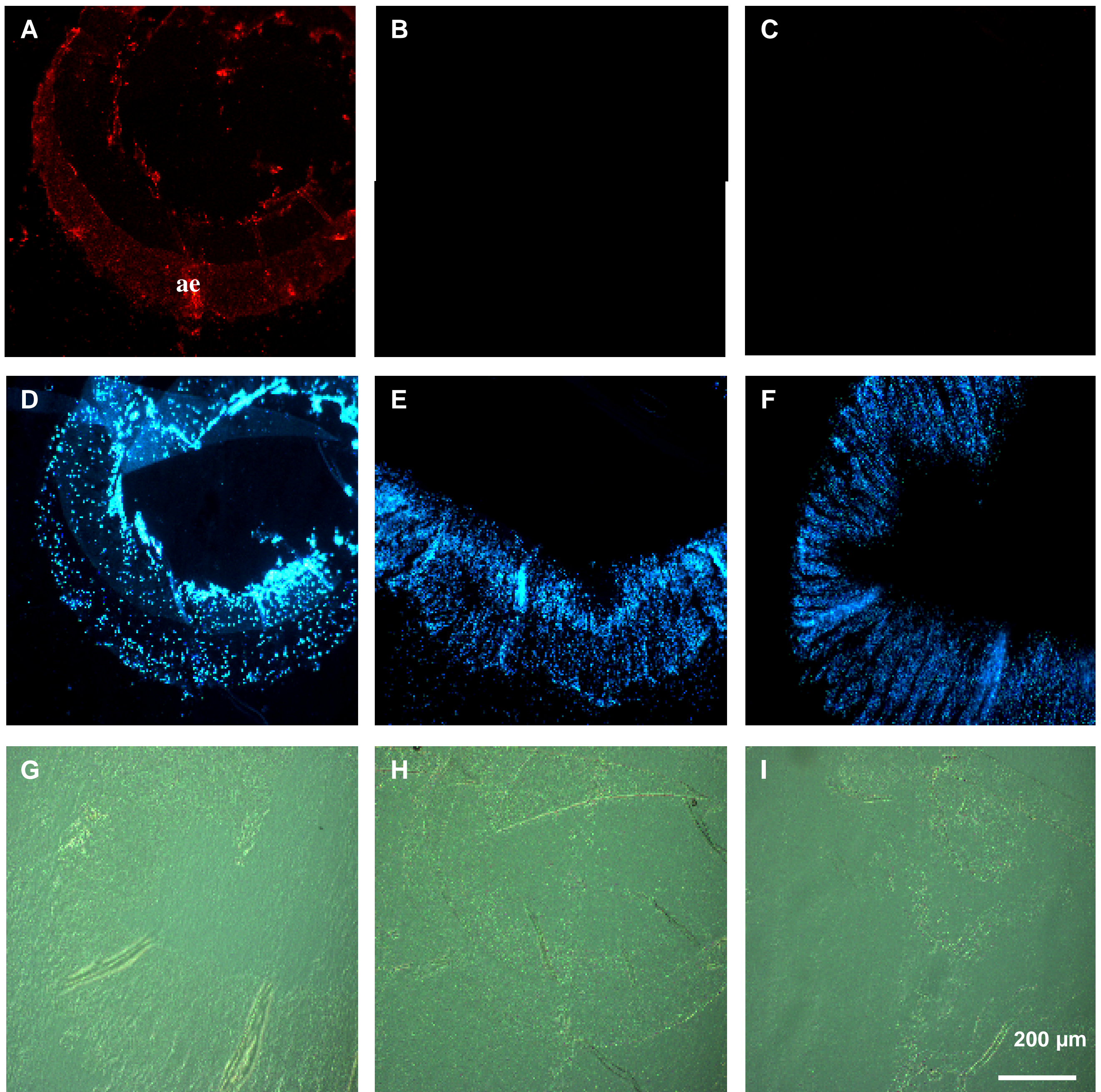


Fig. 57 Labelling of adhesive protein from tube feet of starfish using antibody against adhesive protein from Cuvierian tubules of sea cucumber *Holothuria forskali* (**A** – sections stained with antibody against adhesive protein (1:100 dilution), **B** – sections stained with preimmune serum of adhesive protein (1:100 dilution), **C** – section stained only with secondary antibody , **D, E, F** – section of tube feet stained with DAPI, **G, H, I** – section inspected by light microscopy, **ae** – adhesive epiderms).

6. Discussion

Adhesives are common in many biological systems, and they have great strength and durability. However, their extraction and characterisation can be very challenging, limiting progress in understanding their mechanisms of action. Despite this, many researchers have risen to the challenge, particularly in the extraction of adhesive proteins from invertebrates like mussels and barnacles (e.g. Taylor and Waite, 1997; Kamino *et al.,* 2000). Great success has been obtained with mussels with ten proteins currently known to be involved in adhesion having been identified with nine actually isolated (Silverman and Roberto, 2007). The adhesives employed by echinoderms are, in contrast, poorly understood.

To increase our knowledge about echinoderm adhesion, identification of the proteins involved in the adhesive process has been attempted, as well as isolation and characterisation of these proteins.

Isolation of adhesive proteins from Cuvierian tubules

The study of marine adhesives is a challenging task largely because of their very poor solubility in water (Waite, 1987; DeMoor *et al.*, 2003). The current study has shown that Cuvierian tubules are highly insoluble. Investigating buffers, best results were obtained with two denaturating buffers. Buffers containing SDS (DeMoor, 2003) were not used because SDS could interfere with further tests which were planned. Combining strong buffer solutions with prolonged mechanical stirring resulted in a small amount of Cuvierian tubules being successfully solubilised. The reasons why Cuvierian tubules are so highly insoluble were not investigated as part of the current study but there are few suggestions from other researchers. Aggregation of proteins is largely due to the formation of crosslinks between the different proteins composing the adhesive (DeMoor, 2003). The links were identified as di-DOPA in mussels (see Fig. 58) (McDowell *et al.*, 1999; Burzio and Waite, 2000), and disulfide bonds in barnacles (Naldrett and Kaplan, 1997; Kamino *et al.*, 2000). However, by using the buffer advised by Kamino (2000) for barnacles, solubilisation was not improved, which may suggest that other crosslinks are

involved in tubule adhesive aggregation. Insolubility can be also produced when proteins lose water from their structure and the protein sticks to itself, making it highly insoluble- thus the insolubility could be artefact of the extraction process.

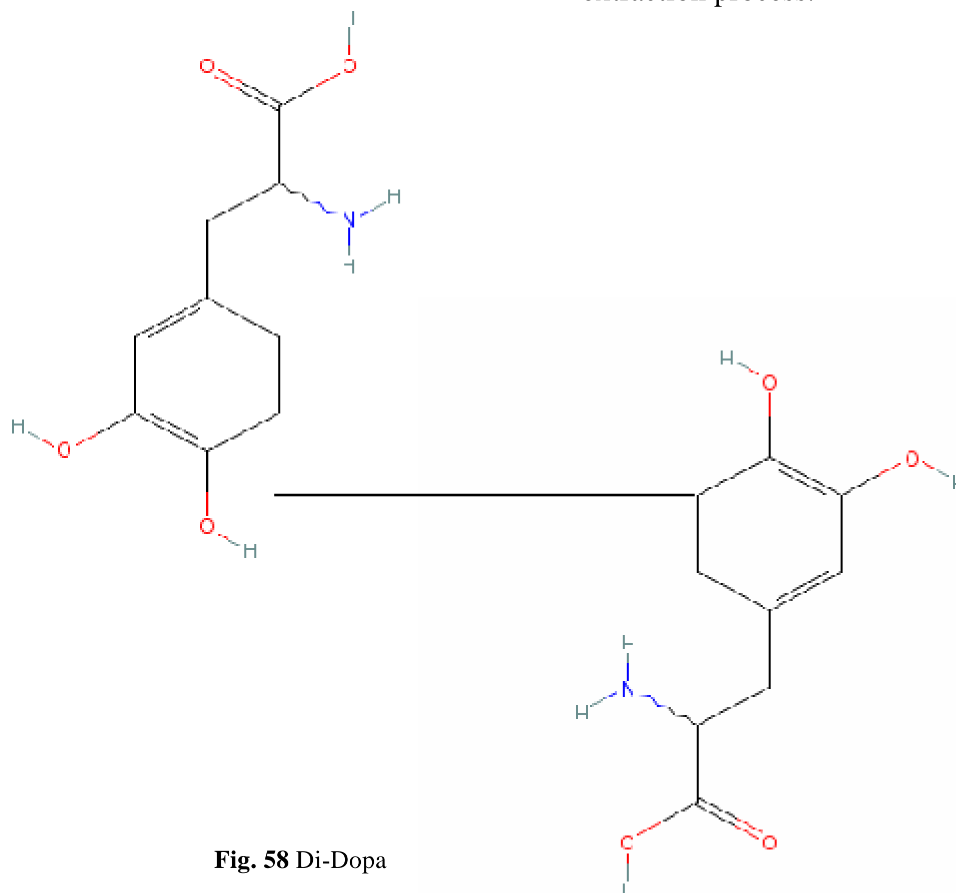


Fig. 58 Di-Dopa

In electrophoretic analysis of tubule print material extract from Cuvierian tubules done by DeMoor *et al.*, 2003 revealed that the extract contained ten different proteins in range 17-220 kDa. In electrophoretic analysis of the Cuvierian tubules extract done in this research revealed several proteins in the range from 10 kDa – 220 kDa were detected. There were a few differences between these two electrophoretic analyses which could be caused by using different extraction methods, particularly different extraction buffers.

When semi-native gels were run, a reduced number of bands were observed, indicating conformational oligomers of some of the proteins or the presence of dimers which could also explain the variations in two electrophoretic analyses.

SDS-PAGE gel was stained with glycoprotein stain to check if there are some proteins which posses carbohydrate group present but only a smear could be observed, with no clear bands visible. A lack of glycoprotein staining suggests that it maybe not be relevant in the extract or the staining is not enough sensitive to detect glycoprotein in the extract.

At this stage it was difficult to recognise which protein is responsible for adhesion. It was decided to combine the existing wide knowledge about mussel adhesives to the poorly understood model of Cuvierian tubules from *Holothuria forskali.* Of the ten proteins that are known and have been identified as being involved in mussel adhesion one of them is Precollagen-D found in the distal thread of the mussel, which contains a silk-fibroin domain.

Silks belong to the class of tensile strength protein fibres which are found in various adhesive systems including spider drag-line (Gosline *et al*., 1999) and silkworms (Bomyx mori) (Chirila *et al.*, 2008). A similar protein has been found in sea urchins (Pearson, 1981). Using an antibody against recombinant silk fibroin protein, it was possible to identify proteins involved in adhesion in Cuvierian tubules of *Holothuria forskali*. The antibodies were used because of the similarity of the silk fibroin sequence with sequences of echinoderms: the similarity was 40% to the purple sea urchin. In Western blots a positive reaction was only obtained with one protein. If the antibody against silk fibroin, protein involve in adhesion have similar epitope to protein in Cuvierian tubule extract is possible to conclude that this protein can be involved in adhesion too.

After identification, isolation of the protein was a challenging task. There have been no adhesive proteins from class Holothuroidea isolated previously. In this research several methods were tested which had previously been applied by other researchers for isolation of non-adhesive protein (Müller *et al.,* 1973; Jacobs and Clad, 1986; Bavington *et al.,* 2004) to get a high quality and quantity of the adhesive protein. Electro-elution was found to produce the highest quantity, which then allowed the protein to be used for the production of antibodies.

The antibody obtained reacted with many extracted proteins on Western blots. Low selectivity of the antibody could be caused by not enough purification of the antigen or

low specify of the antibody. The best conditions for using antibody were developed by their neutralisation.

Electro-eluted protein could not be used for measuring adhesive properties as components of the elution buffer (SDS, glycine) could impair the measurement. To obtain the adhesive protein more conveniently, various ways of isolation were tested. A method for isolation of protein using glass beads was developed. The method is usually used for isolation of DNA (Tai and Tanksley, 1990; Van Burik, 1998) but in this research was developed for isolation of adhesive protein. The best results were obtained with the largest of the beads and at a concentration of 1 M urea. The lowest concentration of urea made the protein precipitate from the solution, allowing them to stick to beads easier. Larger sizes of the beads possess bigger surface to attach which allowed the adhesive protein to stick easier.

Another method for isolation was gel filtration with Sephadex G-50 (Isemura *et al.*, 1973) which was used for purification of adhesive proteins in mussel (Waite, 1983). Sephadex G-50 is suitable to separate proteins in the range of 15 kDa-30 kDa. Adhesive protein was eluted in fractions 6, 7 and 8. After calculation from a formula (Müller, 1973), our protein of interest should elute in fractions 6 and 7. Elution in fraction 8 could be caused by high concentration of the protein and that excess of the extract was applied. When there is excess of extract applied on column, more elution buffer is needed to elute all amount of protein. Result of that is that protein will elute in more fractions than should after calculation. The separation of protein by this method gave good results and a high quantity purified product was obtained.

Immunoprecipitation separated the adhesive protein but in very low quantity and not very good quality. This problem can be caused by interference of the extraction buffer, too high pH or not enough concentration of the protein in extract.

From all the methods of isolation, electro-elution and gel filtration were found the most useful for other applications.

Characterisation of adhesive protein from Cuvierian tubules

Histochemical studies of Cuvierian tubules have a long history; one of the first publications was made in 1868 by Semper, followed by others like Jourdan, 1883, Herouard, 1889 and Müller, 1970.

Using various staining methods, the composition of Cuvierian tubules was analysed. In sections stained with Casson's Trichrome it was possible to conclude that Cuvierian tubules are mainly made up from principally collagen, especially the connective tissue layer which as well was identified by DeMoor (2003). The cytoplasm of adluminal cell in the mesothelium stained red. In stained sections with H&E it was possible to observe collagen, which was stained red by Eosin. Granules cells were stained on purple, they are responsible for tubules elongation and involve in adhesion process (VandenSpiegel, 1987).

Guislain, 1953 found that the Cuvierian tubules are composed from proteins but also from carbohydrates. VandenSpiegel and Jangoux, 1987 found fractions composed of glycoproteins in mucous granules of peritoneocytes in the mesothelium. After analysis, sections were stained with Methylene blue and Azure B. The conclusion was that Cuvierian tubules contain glycoproteins which are stained intensively red in mesothelium layer this can confirm suggestion that glycoprotein stain used for SDS-PAGE was not enough sensitive to recognise glycoprotein in extract. Knowledge about composition of the tubules could help to understand which types of molecules could be involved in process of tubules adhesion.

Localisation of adhesive protein in the tissue sections

DeMoor *et al.*, 2003 localised the origin of tubule print constituents in the tubules by raising polyclonal antibodies against tubule print material. Immunoreactivity was observed in mesothelium layer, which suggested that it is this tissue layer that proteins

involved in adhesion are located. These results were reported before by VandenSpeigel and Jangoux, 1987 and Flammang, 1996.

Immunostaining of the sections of Cuvierian tubules with antibody against adhesive proteins from them, done in this research confirmed that, mesothelium is the tissue layer responsible for the adhesion. It was possible to observe strong immunoreativity in this tissue layer.

DeMoor (2003) found immunoreactivity in the secretory granules of mesothelial granular cells immunoreactivity was recognised. The conclusion was that their secretions make up the bulk of the adhesive material.

To locate more precisely the adhesive protein in the Cuvierian tubules, immunogold labelling and transmission electron microscopy were used. The antisera were strongly immunoreactive in the mesothelium and vacuole, confirming the previous studies showing that the adhesive is located in this layer. There was no labelling with the preimmune sera, confirming that the observed immunoreaction is genuinely between a specific antibody and antigen.

Measurement of adhesion

To understand marine bioadhesion, measurement of the adhesive strength of marine filament and testing under various conditions is helpful. Measurement of the adhesion of Cuvierian tubules under various conditions has previously been studied (Zahn, 1970; Muller, 1972; Flammang, 2003) but measurement of the adhesive strength of Cuvierian tubules extract and isolated protein has not been done. In view of the high insolubility of the Cuvierian tubules, it was necessary to develop methods to extract the adhesives properties before the adhesive properties could be studied and considerable effort was made in this respect.

A protein which can adhere to different surfaces underwater and at low temperature could have a great application in technology and anti-fouling. Paradoxically, a recently developed fouling-release coating was based on marine bio-adhesive possessing strong fouling characteristics. Dalsin *et al.,* 2003 synthesized hybrid molecules by combining a decapeptide derived from Mefp-1 and poly(ethylene glycol) and used these molecules to

modify surfaces. The strategy exploits the adhesive characteristics of the decapeptide to anchor poly(ethylene glycol) onto surfaces, rendering the surfaces resistant to cell attachment *in vitro*. Proteins which have strong adhesive properties can therefore be developed for possible anti-fouling functions. This is one of reasons why various tests on adhesion properties were made.

Dalsian *et al.,* 2002 and Lee *et al.,* 2007 found that the mussel can adhere to any organic or inorganic surfaces. In tests done in this research not all surfaces were suitable for adhesion. The best results were obtained with Teflon, a hydrophobic polymer poly(tetrafluoroethan). There was a very low adhesion of the extraction buffer and strong adhesion from the extract. By measuring adhesion in various dilutions of Cuvierian tubules extract it was possible to find the concentration with the highest adhesion and to develop a standard curve for the extract. The highest adhesion was obtained with 0.5 M urea extract. Müller *et al.,* (1972) reported that urea has an impact on adhesion. Higher concentration of urea inhibits the adhesion; after dilution adhesion increased until optimal point and then it decrease due to the lower concentration of protein. Telfon is known like non-stick surface, so would be expected to show low adhesion. The fact that it shows strong adhesion suggests that the so-called hydrophobic bond process taking place. The adhesive molecules are quite hydrophobic (hence their insolubility) and will tend to displace water from the Teflon and adhesive molecule resulting a force (from the water molecules) that will resist water being pulled into the space between the two Teflon blocks and hence producing a “bond” where in consequence water moving to its lowest possible energy state.

Measurement of adhesion on silicone gave similar results to Teflon surface but adhesion of the buffer could be observed. Silicone has a greater viscosity than Teflon, owing to the size of the particles which can cause the higher adhesion of the extract but as well buffer. Silicon surface is rougher that the Teflon, because of that bonding energy between surfaces higher than on Teflon for the extract as well for the buffer.

The other tested surfaces (glass, iron, gelatine) showed strong adhesion of the buffer, which prevented them from being used as suitable surfaces for measuring adhesion of Cuvierian tubules extract.

Comparing Teflon and glass it is possible to conclude that adhesion is depending on the surface. If the surface is hydrophobic (Teflon) adhesion is strong and there is not interference of the buffer, when surface is hydrophilic (glass) (Krasowska *et al.,* 2003) interaction between the buffer and the surface could be observed.

After testing the adhesion fractions produced by gel filtration, it was possible to observe strong adhesion in fractions where a protein with 18 kDa molecular weight was present, confirming that this protein is responsible for adhesion.

Tests which measured of the effect of the cations and EDTA on adhesion revealed that some of them (cadmium) inhibited adhesion, while EDTA and potassium increased it. Adhesive protein is sensitive for the cations and EDTA. Cadmium has a negative effect on protein, breaking the sulphide bonds between amino acid resulting in the protein losing its structure and adhesive properties (Weber, 1987).

EDTA refers to the chelating agent is widely used to sequester di- and trivalent metal ions (Ca^{2+} and Mg^{2+} for example). EDTA binds to metals via four carboxylate and two amine groups. EDTA forms especially strong complexes with Mn(II), Cu(II), Fe(III), Pb (II) and Co(III) (Holleman and Wiberg E, 2001). From the role which has EDTA it possible to conclude that this amino acid chelated some cations which inhibited adhesion.

Potassium affects solubility of amino acid in aqueous electrolyte soltution (Khoshkbarchi and Vera, 1997) which could caused better solubilisation of adhesive protein and increase their adhesive properties.

Enzymes were tested for their impact on adhesion of Cuvierian tubules, following by Müller and Zahn (1972). Strong inhibition of the adhesion was observed with high concentration of trypsin (1:1, trypsin: extract). With decreasing concentration of the enzyme adhesion increased.

Trypsin is an enzyme which breaks down the protein. This enzyme predominantly cleaves peptide chains at the carboxyl side of the amino acids lysine and arginine, except when they are followed by proline. Adhesive properties of the protein appear to be strongly dependant on its structure. When the concentration of the trypsin is very high, most of the protein molecules break down, changing their primary structure, resulting in loss of their adhesive properties.

Neutralisation of the extract was made by antibody against adhesive protein from Cuvierian tubules. After addition of the antibody to the extract, adhesion was inhibited, most likely because the antibody bound to epitopes on the protein molecule that are either directly involve in adhesion or the bound antibody interferes stereochemically with the adhesion between the molecule and the surface.

Neutralising the adhesive protein by application of trypsin or antibody could be useful in designing antifouling compounds. Knowledge about blocking adhesion can be helpful in understanding adhesive processes and inhibit them when necessary.

Silk fibroin protein, which is involved in adhesion of mussels threads, was tested for its adhesive properties to confirm that the protein has adhesive properties and that it was the right decision to use antibody against it for identification of adhesive proteins in Cuvierian tubules. Silk fibroin adheres in a similar but stronger manner (though not significantly differenct) than the Cuvierian tubule protein extract, which may be due to the fact that this protein is involved in permanent adhesion, while Cuvierian tubules are involved in temporary adhesion (Flammang, 1996). Silk fibroin and the adhesive protein from Cuvierian tubules have high tensile strength of their collagenous core (Waite, 1998; VandenSpiegel and Jangoux, 1987; Hamel and Mercier, 2000). Silk fibroin belongs to the proteins which remain their adhesive properties, whereas adhesive forces of the protein from Cuvierian tubules diminish with time.

Urea has a different impact on mussel extract than Cuvierian tubules extract, the maximum adhesion was obtained when urea concentration was 4 M. Above this concentration, adhesion decreased. Various effects of different factors can suggest that the structures of adhesive proteins in Cuvierian tubules are not similar to structures of the proteins in mussel extract and they interfere with the buffer differently but both extract react with the same antibody, which may suggest that adhesive proteins have these same epitope.

Labelling adhesive protein in mussels threads using antibody against adhesive protein from Cuvierian tubules

Features of the byssus in mussels include: a root attached to the byssal retractor muscle: a stem extending from the root and individual byssal threads which are attached to overlap cuffs of the root (see Fig. 59). The byssal thread consists of a flexible, collagenous inner core surrounded by hardened, cured polyphenolic protein (Silverman and Roberto, 2007 Tamarin *et al.,* 1976). After treatment of mussel byssus with antibody against Cuvierian tubule protein, a positive reaction was obtained in the area of the distal thread, where Precollagen-D with the silk fibroin domain is present (Fig. 51). High immunoreactivity with the byssus threads proved that antibody can recognise adhesive protein in mussel byssus, and confirmed that the isolated protein from Cuvierian tubules has an immunological similarity with silk fibroin from mussel. Proteins which have similar amino acid composition will give positive reaction with antibody. There was no immunoreactivity with preimmune serum which confirms that for the positive reaction witnessed there has to be antibodies present that interact with specific adhesive proteins.

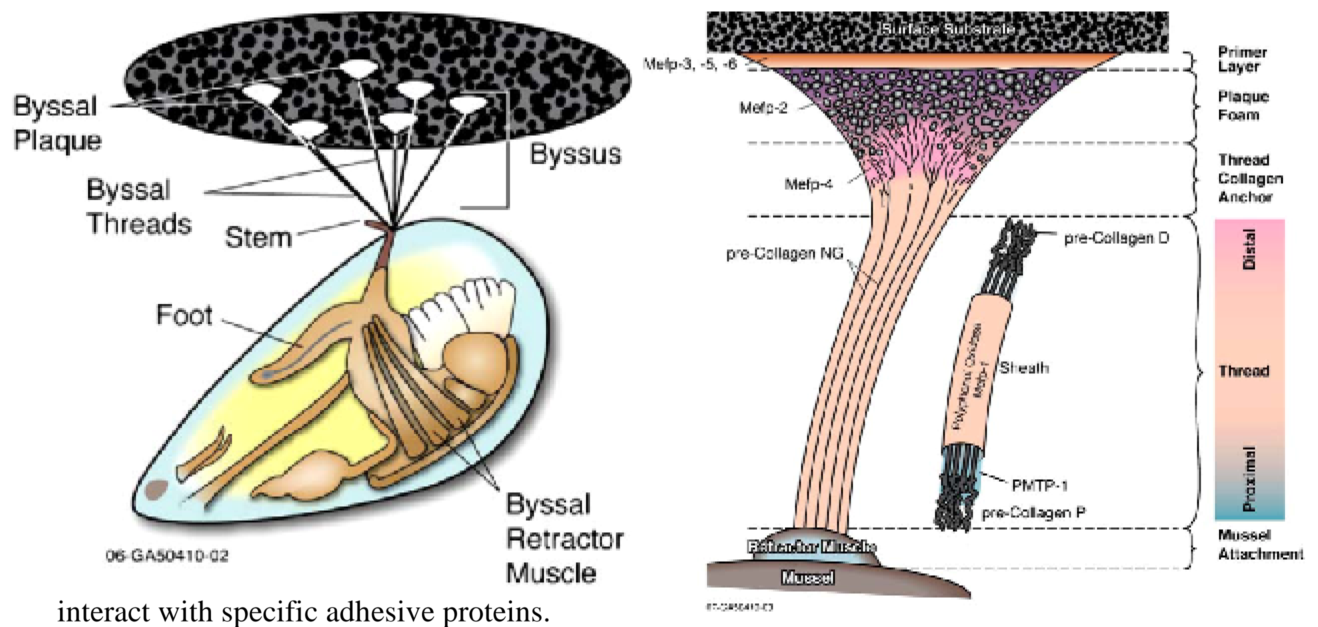


Fig.59 Anatomy of mussel and byssus and thread structure with localisation of adhesive proteins (Silverman and Roberto, 2007).

Isolation of adhesive protein from the tube feet and footprint of starfish *Marthasterias glacialis*

Tube feet and footprints of starfish have captured the interest of various researchers including Smith, 1937; Nichols, 1966 and Flammang, 1996.

Footprints in echinoderms consist of a sponge-like material deposited as a thin layer on the substratum (Thomas and Hermans, 1985; Flammang, 1996 and Flammang, 1998). Previous studies on starfish podia and their footprints have, by necessity, concentrated largely on physiological and anatomical research.

Isolation of the adhesive protein(s) from either tube feet or the footprints has not been previously reported. The starting point of this study was to choose the most suitable species from available starfish. Collection of adhesive protein caused problems because it was very difficult to obtain pure footprints in any quantity. Collection of protein by scraping the glass with the footprints as proposed by Flammang (1998) was used but the quantities obtained were insufficient for characterisation work. An alternative approach was to collect tube feet by cutting them from the animal. While this gave plentiful tissue, homogenisation of the specimen was quite difficult. Several buffers were tested such as proposed by Hamwood *et al.*, 2002 but, even if the homogenisation was successful, the extract was not suitable for further analysis as precipitation often occurred (i.e. with guanidine hydrochloride). The buffer composed of urea and Tris gave the best results of the buffer tested and proved useful for further analysis. SDS-PAGE of the tube feet extract showed a smear which could be caused by incomplete solubilisation of the specimen or contamination from the environment, prior purification, was needed.

Several proposed stains for SDS-PAGE were tested. Satisfying results were obtained with silver stain but there was a high background which could be caused by presence of carbohydrates as reported by Flammang, 1998. High background was eliminated by using Imperial stain; the sensitivity of this stain was comparable to silver stain. In the lane of the footprint only one band was observed. The conclusion was that this may be the protein involved in adhesion because only the footprint is left after starfish adhesion, so any other proteins should not be in the starfish footprints. Referring to the duo-gland hypothesis the protein in the footprint can be adhesive or de-adhesive

but after testing adhesive properties of the footprint using AFM where adhesion of the footprint was so strong that stick to cantilever and enable any force measurement, it is possible to conclude that this protein is adhesive.

One way to isolate the protein was digestion of the band and then use HPLC to check for the presence of the protein in the digestion solution. The protein was also isolated and purified on HPLC straight from the homogenised footprints extract. As proposed by Bavington *et al.,* 2004, a size exclusion column was used for the determination of the size of the protein. Extract of footprints loaded on the column showed a peak at around 25 min, giving a calculated expected molecular mass of 50 kDa. After injection of the digestion mixture, no peak was obtained, possibly caused by insufficient quantity of proteins applied on the column or because the digestion buffer interfered with the column. To found out what caused the problem, absorbance of the mixture was measured at 260 nm (Lyne, 1957) to detect any significant presence of protein. The mixture showed low absorbance, allowing the conclusion that the problem was caused by a low quantity of protein in the sample.

A reverse phase column used for purification of sample also showed the presence of the protein in fresh footprint extract around 25 min but no significant of the protein in digestion mixture confirming the prior statement.

Characterisation of adhesive secretions from footprint and tube feet of starfish

Perpet and Jangoux, 1973 have published a detailed histochemical study of the podia *Asterias rubens* (Echinodermata, Asteroidea) and suggest that the footprint is composed of carboxylated protein and glycosaminoglycans (long unbranched polysaccharides consisting of a repeating disaccharide unit) as well as un-carboxylated proteins. Chaet, 1965 and Flammang, 1994 proposed that footprint is composed of proteins and glycosaminoglycans but no lipids. Flammang *et al.,* 1998 present a biochemical composition of the footprints *Asterias rubens* and showed that the footprint is mainly composed of proteins, but carbohydrates and lipids are present as well. The presence of lipid is questionable. This fraction might not detected by classical histochemical methods or its presence could be contamination of the footprint (Flammmng *et al.,* 1998).

Various stains which detect different components composition were used to analyse the footprint from *Marthasterias glacialis.*

Alcian blue stains glycosaminoglycans and this stain appeared very strongly in the footprint. Presence of protein in the footprint was proved using the stains Ponceau/Acid fuchsine and Coomassie Blue: Footprints were strongly stained with both stains.

To detect the presence of lipids Oil Red O was used; this stain is suitable for staining lipid derivates. Oil Red O did not stain any parts of the footprint so the conclusion was that there is no lipid in the footprint confirming that results obtained by Flammang *et al.,* 1998 was probably contamination of the sample.

Physiological studies of tube feet of starfish have been performed by many researchers and they agree that the structure of the podia is constant for all echinoderms and composed of an inner endothelium enclosing the water vascular system and an outer epithelium, with a connective tissue layer and a nerve plexus between them (Kawaguti, 1964; Nichols, 1966; Florey and Cahill, 1977; Flammang and Jangoux, 1992 and Flammang, 1996). Two stains H&E and Casson's Trichrome were used to confirm composition of the tube feet from *Marthasterias glacialis* and to check biochemical composition. H&E stain showed that tube feet are mainly composed of collagen; in the outer epithelium the strongest staining is red from Eosin, which stains proteins confirming presence of this type of molecules in the tube feet. In the connective tissue it was possible to observe cells stained purple by Haemotoxylin. Casson's Trichrome confirmed that collagen is the main component of tube feet, which was stained blue by aniline blue which is constituent of Casson's Trichrome. Only the epidermis stained red by Acid fuchsine, which responsible for staining the cytoplasm in cells. Cells are at a high density in this tissue layer (DAPI stain in immunostaining confirmed this).

AFM analysis of the footprint of *Marthasterias glacialis* proved that its surface had the appearance of a net, which was shown in *Asterias rubens* by Flammang *et al.,* 1994 and earlier by Thomas and Hermans, 1985. Footprints appear like disc-shaped films, made up of material having a sponge like appearance, with numerous holes in the matrix. It is interesting that the adhesive secretions of some marine invertebrates using permanent adhesion have also a spongy organisation like byssal plaque matrix of mussels (Benedict and Waite, 1986; Waite, 1986) and the basal cement of barnacles (Naldrett, 1993).

It has been shown that there is more than one type of secretory cell present in the tube foot. One secretory cell releases adhesive secretion but the function of the rest secretory cell is still under discussion. Hermans, 1985 and Flammang, 1994 suggest that a second type of secretory cells (ciliated) produce the de-adhesive secretion. McKenzie, 1988 suggested instead that ciliated cells are nervous cells involved in control of the secretion.

The net like structure of the footprint reflects the topography of the tube foot disc. Further AFM analysis was planned to measure adhesion on surface of the net and inside the net holes to check if there are some different adhesion forces, but it was not possible to work with fresh material which stuck to cantilever and this making further measurement impossible.

To better study the surface of the footprint, an optical profiler (Wyko NT1100) was used which showed how complicated the surface of the footprint is. The thickness of the footprint varies across the whole surface and the surface of the footprint again looks like the net. The image of the footprint obtained by this equipment looks like as if the tube foot was pulled from the surface using mechanical forces because of the irregular thickness on the surface. If there would be adhesive-de-adhesive system the footprint thickness on all surfaces would more likely to be similar. The speculation can be that possible there is no de-adhesive secretion and de-adhesion of the tube foot is made by mechanical forces how as proposed by McKenzie, 1988.

Antibody against adhesive protein from Cuvierian tubules was used to identify the location of adhesive protein in starfish tube feet and to localise it in sections of the tube feet. In Western blot it was possible to observe two bands, one at 50 kDa and second at 100 kDa. Only the 50 kDa protein from tube feet was present in the footprint. It is thus probable that this protein is responsible for adhesion. Immunoreactivity of the antibody in the outer epidermis confirmed that in this area of the tube feet the adhesive protein is present. Preimmune serum did not show any immunoreactivity.

Conclusions:

Identification and isolation of the adhesive proteins was the first step to explore echinoderm adhesion. Higher purification of adhesive protein is needed for other

purposes; 2D would help to find out if there is only one protein behind the identified bands on SDS-PAGE. Immunohistochemical location of the adhesive protein at TEM level would show which cells are responsible for realising the adhesive. Identifying the chemical groups which make the adhesive organs high insoluble would help to understand better the process of adhesion. Measurement of adhesion using AFM could help resolve the discussion about adhesion in starfish. More studies must be done, on a molecular level. Identification of the genes encoding the adhesive proteins and synthesis recombinant protein would be first step in commercialising new adhesives as it would allow strong patents to be developed around described proteins.

The production of underwater adhesives that mimic the properties of echinoderms and other marine invertebrates remains a challenge and the current researchers had to overcome many challenges.

The very first step in this research-the homogenisation of adhesive organs-was main barrier to the success of the research. After this was overcome allowing the solubilisation of the specimens, identification of the adhesive protein was made using the much better developed, knowledge about adhesive proteins in mussels. Various isolations methods were tested to get the best quantity and quality adhesive protein. A new method using glass beads for the isolation of protein was developed. Antibodies against adhesive protein were raised and used for localisation the protein in tissue sections by fluorescence microscopy and TEM. Best conditions to use the antibodies by their neutralisation were developed. Special equipment for measure adhesion of extracts was constructed and used for various measurements. Tests of adhesive properties in different conditions were done to check in which environments adhesion is the highest as well how is possible to block the adhesion. These tests can be useful for technological application of the adhesive. Characterisation of adhesive organs was made using traditional stains as well the new technology tools such as AFM, Wyko.

The main findings in this study are solubilisation of tissue with high insolubility and identification of adhesive proteins in extracts. Isolation first adhesive protein from Holothria forskali by known methods and developed new method for isolation by glass beads was successful. Construction of machine which measure adhesive properties of the liquid was challenging. Measurement adhesion of the extract under various conditions

could have application in technology. Characterisation organs responsible for adhesion by histochemistry would help understand composition of adhesive organs. Characterisation of adhesive proteins in organs by immunohistochemistry and immunocytohistochemistry could help to find out localisation of protein. Images of the starfish footprint done by tools like AFM, Wyko showed an opportunity to use new technology to characterised biological specimens. Similarities of the adhesive protein from Cuvierian tubules with protein involve in adhesion in mussels and starfish could make new approach in echinoderm studies.

Hopefully this study improves knowledge about echinoderms adhesion.

7. Summary

The main goal in this research was to isolate and characterise adhesive proteins from two species of echinoderms (*Holothuria forskali* and *Marthasterias glacialis* drawn from two classes: the Holothuroidea and the Asteroidea).

Adhesion is a way of life in echinoderms. All the species from this phylum use adhesive secretions for various functions. Biotechnological interest in echinoderm bioadhesives cover two broad fields of: design of water-resistant adhesives and development of new antifouling strategies. The research on echinoderms was carried out in Integrin Advanced Biosystems in Oban (Scotland) and at the Institute for Physiological Chemistry and Patobiochemistry of University of Mainz (Germany). The main task in understanding the process of adhesion was to recognise the molecules responsible for adhesion, including their isolation and characterisation. From animals collected in Scotland (starfish) and Croatia (sea cucumber), adhesives organs were removed.

Homogenisation of the specimens was challenging task because it was difficult to find buffers for successful solubilisation of the tissue. After extraction, identification of the adhesive molecules from Cuvierian tubules (sea cucumber) was made by using the antibody against protein involve in adhesion from mussels on a Western blot and highlighting the protein in the extract of footprints from starfish by several stains on SDS-PAGE. Using an antibody against recombinant silk fibroin protein, it was possible to identify proteins involved in adhesion in Cuvierian tubules of *Holothuria forskali*. The antibodies were used because of the similarity of the silk fibroin sequence with sequences of echinoderms: the similarity was 40% to the purple sea urchin.

An adhesion molecule in Cuvierian tubules with size of 18 kDa was identified as was a larger protein from the footprint of starfish, which was in the order of 50 kDa. To get the best quantity and quality of the adhesive proteins various methods were tested for isolation such as electro-elution, immunoprecipitation, gel filtration, HPLC. A new method for isolation using glass beads was developed. To localise adhesive protein in organs, antibodies against adhesive protein from Cuvierian tubules of sea cucumber were raised. Adhesive protein in Cuvierian tubules (sea cucumber) is localised in mesothelium, in tube feet (starfish) strong immunoreactivity was observed in the epidermis.

Characterisation of adhesive proteins was made using a surface-imaging tool AFM; a surface profiling instrument (Wyko NT 1100 in collaboration with VEECO, Cambridge) and electron microscopy. To quantify the adhesive properties several of the various proteins, tests were made under various conditions such as: different solvents, surfaces, with additions of cations and enzymes, various concentrations of extract.

Isolations of the adhesive protein will enable the next target to be addressed which involves finding encoding genes and to produce recombinant adhesive protein which can be commercialised. This study could help to develop new water resistant adhesive proteins from different sources than are used now.

8. Summary (germ)

Das Hauptziel dieser Arbeit war, Adhäsionsproteine aus zwei Arten von Echinodermen (*Holothuria forskali* (Holothuroidea) und *Marthasterias glacialis* (Asteroidea) zu isolieren und zu charakterisieren.

Adhäsion ist ein wichtiger Bestandteil der Lebensweise von Echinodermen (Stachelhäutern). Alle Arten dieses Stammes nutzen Adhäsion-Sekretion zu verschiedenen Zwecken. Das Interesse an diesen biologischen Klebstoffen umfasst zwei Gebiete der angewandten Forschung: Die Entwicklung wasserresistenter Klebstoffe und die Gestaltung neuer Antifouling-Strategien.

Die Untersuchungen an Stachelhäutern wurden in der Firma Integrin Advanced Biosystems in Oban (Schottland) und am Institut für Physiologische Chemie und Pathobiochemie der Universität Mainz (Deutschland) durchgeführt. Um den Prozess der Haftung zu verstehen, war die Entdeckung der Adhäsionsmoleküle, die in den genannten Organismen für die Adhäsion verantwortlich sind, die wichtigste Aufgabe, und in Anschluß Isolierung und Charakterisierung. Die Tiere wurden in Schottland (Asteroidea, Seesterne) und Kroatien (Holothuroidea, Seegurken) gesammelt und die Ambulakralfüßchen bzw. Cuvierschen Organe wurden vor Ort entfernt und bis zur weiteren Verarbeitung gelagert. Die Homogenisierung der Proben stellte eine Herausforderung dar, da zunächst geeignete Puffer zum Auflösen der Gewebe gefunden werden mussten. Nach der Extraktion wurden die Klebstoff-Moleküle der Cuvierschen Organe (der Seekurken) im Western Blot identifiziert; hierfür wurde ein Antikörper gegen Klebstoff-Proteine aus Muscheln eingesetzt. Das Klebe Protein der Seesterne wurde in Extrakten der Fußabdrücke der Tiere über verschiedene Färbungen von SDS-Polyacrylamidgelen nachgewiesen.

Das Adhäsionsmolekül aus den Cuvierschen Organen der Seegurken hat eine Größe von 18 kDa, jenes im Fußabdruck des Seesterns 50 kDa. Um bestmögliche Qualitäten und Quantitäten der Klebstoff-Proteine zu erhalten, wurden verschiedene Methoden zur Isolierung wie Elektro-Elution, Immunopräzipitation, Gel-Filtration und HPLC gestestet. Zur Lokalisierung der Proteine (in den Organen) wurden Antikörper gegen das Klebstoff-Protein aus den Cuvierschen Organe der Seegurken hergestellt. Das

Klebstoff-Protein der seegurken ist im Mesothel lokalisiert; in den Ambulakralfüßchen der Seesterne wurden starke Immunreaktionen in der äußeren Epidermis beobachtet. Die Charakterisierung der Klebstoff-Proteine wurde mithilfe von AFM, Wyko NT-1100 (Zusammenarbeit mit Veeco, Cambridge) und mit von Elektronenmikroskopie durchgeführt. Um aufgrund ihrer adhäsiven Eigenschaften mögliche Anwendungen der Proteine zu testen, wurden mehrere Versuche unter variablen Bedingungen wie z.B. verschiedene Lösungsmittel und Oberflächen, An- und Abwesenheit von Kationen und Enzymen und verschiedene Konzentrationen der Extrakte durchgeführt. Die vorliegende Studie könnte zur Entwicklung von neuartigen, wasserresistenten kommerziellen Klebstoffen aus andersartigen Quellen als bisher beitragen.

9. References

Abarzua S, Jakubowski S (1995) Biotechnological investigation for the prevention of biofouling. I. Biological and biochemical principles for the prevention of biofouling. *Mar. Ecol. Prog. Ser.* 123:301 – 312

Abbott A (1990) Bioadhesives: potential for exploitation. Sci. Progress 74: 131 – 146

Albala DM (2003) Fibrin sealants in clinical practice. Cardiovascular Surg. 11(S1):5 – 11

Barker MF (1977) Observations on the settlement of the brachiolaria larvae of Stichaster australis (Verrill) and Coscinasterias calamaria (Gray) (Echinodermata: asteroida) in the laboratory and the shore. *J. Exp. Mar. Biol. Ecol*. 30:95 –.108

Bavington CD, **Lever R**, **Mulloy B**, **Grundy MM**, **Page CP**, **Richardson NV**, **McKenzie (2004)** Anti-adhesive glycoproteins in echinoderm mucus secretions. *Comp. Biochem. Physiol., Biochem. Mol. Biol.*. 2004 Dec; 139(4):607 – 17.

Becker K (1993) Attachment strength and colonization patterns of two macrofouling species on substrata with different surface tension (in situ studies). *Mar. Biol.* 117/2; 301 – 309, Springer Berlin/ Heidelberg

Benyus JM (2002) Biomimicry: Innovation Inspired by Nature. Publisher: Harper Perennial (September 17)

Benedict CV, Waite JH (1986) Composition and ultrastructure of the byssus of *Mytilus edulis. J. Morph.* 189, 261 –.270

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 72:248 – 254

Burzio LO, Burzio VA, Silva T, Burzio LA, Pardo J (1997) Enviromenta; bioadhesion: themes and applications. *Curr. Opin. Biotechnol.* 8:309 – 312

Burzio LO, Gutierrez E, Pardo J, De la Fuente E, Brito M, Saez C (1990) Bioadhesives: A biotechnological opportunity. *Arch. Biol.* Med Exp. 23: 173 – 178 (1990) Chile

Burzio LA and Waite JH (2000) Cross-linking in adhesive quinoproteins: studies with model decapeptides. Biochemistry 39:11147 – 11153.

Callow ME, Callow JA (2002) Marine biofouling: a sticky problem. *Biologist* 49:1–5

Cameron JL, Fankboner PV (1984) Tentacle structure and feeding processes in life stages of the commercial sea cucumber *Parastichopus californicus* (Stimpson). *J. Exp. Mar. Biol. Ecol*. 81:193 – 209

Cawthorne DF, Davenport J (1980) The effects of fluctuating temperature, salinity and aerial exposure upon larval release in *Balanus balanoides and Elminius modestus. J. Mar. Biol. Assoc. UK* 60:367 – 377

Chadwick HC (1907) *Antedon*. Liverpool Marine Biological Committee Memoir 15:47

Cheat AB (1964) Invertebrates adhering surfaces: Secretions of the starfish, *Asterias forbesi* and the coelenterate, *Hydra pirardi. Ann. N.Y. Acad. Sci.* 118, 921 – 929

Chirila TV, Barnard Z, Zainuddin HDG, Schwab IR, Hirst LW (2008) Bombyx mori silk fibroin membranes as potential substrata for epithelial constructs used in the management of ocular surface disorders. *Tissue Eng. Part A*.

Crisp DJ, Meadows PS (1962) The chemical basis of gregariousness in cirripedes. *Proc. R. Soc.* B156: 500 – 520

Crisp DJ (1973) Mechanism of adhesion of fouling organism. In: Ackew RF, Brown BF DePalma J R & Iverson WP (eds) *Proc 3rd Int Cong Mar Corrosion and Fouling.* Northwestern University Press , Evanston , pp 691 – 708

Crozier WJ (1915) A note on the physiology of the Cuvierian organs of *Holothuria captive. Am. J. Physiol. 36,* 196 – 202 (1915)

Dalsin JL, Hu B, Lee B, Messersmith PB (2003) Mussel adhesive protein mimetic polymers for the preparation of nonfouling surfaces. *J. Am. Chem. Soc.* 2003, 125, 4253 – 4258 9 4253

Danscher G (1981) Light and electron microscopic localization of silver in biological tissue. Histochemistry 71:177 – 186.

DeMoor S, Waite H, Jangoux M, Flammang P (2003) Characterization of the adhesive from Cuvierian tubules of the sea cucumber *Holothuria forskali* (Echinodermata, Holothuroidea). *Mar. Biotechnol.* 5, 45 – 57, 2003

Despain RR, De Vries KL, Luntz RD, Williams ML (1973) Comparison of the strength of barnacle and commercial dental cements. *J. Dent. Res.* 52(4): 674 – 679, 1973

Determann H (1969) Gel chromatography. Berlin – Heidelberg- New York: Springer-Verlag

Endean R (1957) The Cuvierian tubules of *Holothuria leueospilata. Q. J. microsc. Sci. 98,* 455 – 472

Firth FE (1969) The encyclopedia of marine resources. p.60 – 62 Van Nostrand Reinhold Company, New York

Flammang PC, Jangoux M (1992) Functional morphology of the locomotory podia of *Holothuria forskali* (Echinodermata, Holothuroidea). *Zoomorphology* 111:167 – 178

Flammang P, Demeulenaere S, Jangoux M (1994) The role of podial secretions in adhesion in two species of sea stars (Echinodermata). *Biol. Bull. Mar. Biol. Lab., Woods Hole* 187, 34 – 47

Flamang P (1996) Adhesion in echinoderms. In *Echinoderms studies,* vol. 5. (Jangoux M., and Lawrence L. M.), pp 1 – 60 Rotterdam: Balkema

Flammang P, Gosselin P, Jangoux M (1998) The podia, organs of adhesion and sensory perception in larvae and postmetamorphic stages of the echinoid *Paracentrotus lividus* (Echinodermata). Biofouling 12:161 – 171

Flammang P, Michel A, Cauwenberge AV, Alexandre H, Jangoux M (1998) A study of the temporary adhesion of the podia in the sea star *Asterias Rubens* (Echinodermata, asteroidea) through their footprints. *J. of Experimen. Biol.,* Vol 201, Issue 16 2383-2395, print by Company of Biologists

Flammang P, Ribesse J, Jangoux M (2002) Biomechanics of adhesion in sea cucumber Cuvierian tubules (Echinodermata, Holothuroidea). *Integr. Comp. Biol.* 42:1107 – 1129

Flammang P, Santos R, Haesaerts D (2005) Echinoderm adhesive secretions: From experimental characterization to biotechnological applications. In Progress in Molecular and Subcellular Biology, Marine Molecular Biotechnology, Echinodermata (ed. V. Matranga), pp.199 – 218. Berlin: Springer-Verlag

Florey E, Cahill MA (1977) Ultrastructure of sea urchin tube feet. Evidence for connective tissue involvement in motor control. *Cell Tissue Res.* 177: 195 – 214

Gislen T (1924) Echinoderm studies. *Zool Bidr* Uppsala. 9:270 – 295

Gorb S, JiaoY, Scherge M (2000) Ultrastructural architecture and mechanical properties of attachment pads in Tettigonia viridissima (Orthoptera, Tettigoniidae). *J. Comp. Physiol.* A186, 821– 831.

Gosline JM, Guerette PA, Ortlepp CS (1999) The mechanical design of spider silks: from fibroin sequence to mechinal function. *J. Exp. Biol.* 1999 Dec; 202(Pt 23): 3295 – 30

Gosselin P, Jangoux M (1998) From competent larva to exotrophic juvenile: a morphofunctional study of the perimatamorphic period of *Paracentrotus lividus* (Echinodermata, Echinoidea). *Zoomorphology* 118:31– 43

Grenon JF, Walker , G. (1978) The histology and histochemistry of the pedal glandular system in two limpets, *Patella vulgate* and *Acmaea tessulata* (Gastropoda: Prosobranchia). *J. of the Mar. Biol. Assiocat. of the United Kingdom* 58 803 – 816

Grenon JF, Walker G (1980) Biochemical and rheological properties of the pedal mucus of the limpet ,*Patella vulgate* L. *Comp. Biochem. and Physiol.* 66B, 451 – 458

Guislain R (1953) Recherches histologiques sur les canaux de Cuvier de *Holothuria impatiens* Forskal. *CR Soc. Biol.* Paris *147,* 1254 – 1256

Haeserts D, Jangoux M, Flammang P (2003) Study of the perimatamorphic period of the sea star *Asterias rubens* by scanning electron microscopy. In: Feral JP, David B Echinoderm research 2001. AA Balkema, Lisse, pp 155 – 159

Hamel JF, Mercier A (2000) Cuvierian tubules in tropical holothurians: Usefulness and efficiency as a defense mechanism. *Mar. Fresh. Behav. Physiol.* 33:115 – 139.

Hamwood TE, Cribb BW, Halliday JA, Kearn GC, Whittington ID (2002) Preliminary charactersation and extraction of anterior adhesive secretion in monogenean (platyhelminth) parasities. *Folia parasitol.* 49: 39 – 49

Harlow E, Lane D (1998) Immunoglobulins/ Laboratory manuals CSHL Press

Hermans CO (1983) The duo-gland adhesive system. *Oceanogr. Mar. Biol. Ann. Rev.21:*281 – 339

Herouard E (1889) Recherches sur les holothuries des c6tes de France. *Archs. Zool.* Exp. gn. 7, 535 – 704

Hickman CP, Roberts LS, Larson A, Anson H, Eisenhour DJ (2006) Integrated Principles of Zoology; McGraw Hill; New York, (Thirteen edition)

Holleman AF, Wiberg E (2001). Inorganic Chemistry. San Diego: Academic Press. ISBN 0-12-352651-5.

Isemura M, Zahn R, Schmid K (1973) A new neuran minic acid derivative and three types of glycopeptides isolated from the Cuvierian tubules of the sea cucumber *Holothuria forskali Biochem. J.* 131, 509 – 521 509 Printed in Great Britain

Jacob E, Clad A (1986) Electroelution of fixed and stained membrane proteins from preparative sodium dodecyl sulfate-polyacrylamide gels into a membrane trap. *Anal. Biochem.* 154, 583 – 589

Jangoux M, Lawrence JM (1996) Echinoderms studies Volume 5 Balkema, Rotterdam, Brookfield

Jourdan M (1883) Recherches sur l'histologie des holothuries. *Ann. Mus. Hist. Nat.* Marseille *1 (6),* 1-64

Kamino K, Inoue K, Maruyama T, Takamatsu T, Harayama S, Shizuri Y (2000) Barnacle cements proteins. Importance of disulfide bonds in their insolubility. *J. Biol. Chem.* 275:27360 – 27365

Kawaguti S (1964) Electron microscpic structure of the podial wall of an echinoid with spetial reference to the nerve plexus and the muscle. *Biol. J. Okayama Univ.* 10:1 – 12

Khandeparker L, Anil AC (2007) Underwater adhesion: The barnacle way. *Inter. J. of Adhesion & Adhesives* 27 (2007) 165 – 172

Khoshkbarchi MK , Vera JH (1997) Effect of NaCl and KCl on the Solubility of Amino Acids in Aqueous Solutions at 298.2 K: Measurements and Modeling *Ind. Eng. Chem. Res.*, 36 (6), 2445 – 2451

Krasowska M, Krzan M, Malysa K (2003) Bubble collisions with hydrophobic and hydrophilic surfaces in α-Terpineol solutions. *Fizykochem. Prob. Mineral.* 2003, Vol. 37, s. 37 – 50

Layne E (1957) Spectrophotometric and turbidimetric methods for measuring proteins. *Met. in Enzym. 3:* 447 – 455

Lawrence J M (2001) Function of eponymous structures in echinoderms: A review. Can. *J. Zool*. 79:1251 – 1264.

Lee H, Scherer NF, Messersmith PB (2006) Single-molecule mechanics of mussel adhesion. Proc Natl Acad Sci U S A; 103(35): 12999 – 13003 *App. Physic,l Scien., Biochem.*

Lee H, Lee B, Messersmith P (2007) A reversible wet/dry adhesive inspired by mussels and geckos *Nature* Vol. 448

Lowry OH, Rosbrough NJ, Farr AL, Randall RL (**1951)** Protein measurement with the Folin phenol reagent*. J. Biol. Chem*. 193:265 – 275

Martin GG (1978) Ciliary gliding in lower invertebrates. *Zoommorphologie* 91, 249 – 261

Matsumara K, Nagano M, Kato-Yoshinaga Y, Fusetani N (1996) A larval settlement – inducing protein complex (SIPC) of the barnacle, *Balanus amphitrite.* In: Clare A S (ed) *Settlement and Metamorphosis of Marine Invertebrate Larvae.* University of Plymouth, Plymouth

McDowell LM, Burzio LA, Waite JH, Schafer J (1999) Rotational echo double resonance detection of cross links formed in mussels byssus under high-flow stress. *J. Biol. Chem.* 274:20293 – 20295

McKenzie JD (1987) The ultrastructure of the tentacles of eleven species of dendrochirote holothurians studied with special reference to surface coats and papillae *Cell Tissue Res.* 248: 187 – 199

McKenzie JD (1988) The ultrastructure of tube foot epidermal cells and secretions: Their relationship to the duo-glandular hypothesis and the phylogeny of the echinoderm classes. In: Paul, C. R. C& Smith, A. B. (eds) *Echinoderm Phylogeny and Evolutionary Biology:* 287 – 298. Clarendon Press, Oxford

McKenzie JD (1992) Comparative morphology of crinoid tube feet. In: ScaleraLiaci L, Canicatti C *Echinoderm Research 1991*: 73 – 79. Balkema, Rotterdam

Merril CR, Dunau ML, Goldman D (1981) A rapid sensitive silver stain for polypeptides in polyacrylamide gels. *Anal. Biochem.* 110(1), 201 – 7.

Merril CR, Pratt ME (1986) A silver stain for the rapid quantitative detection of proteins or nucleic acids on membranes or thin layer plates. *Anal. Biochem.*. 156(1), 96 – 110.

Mines G (1912) Note on the mechanism of discharge of the Cuvierian organs of *Holothuria nigra.* Q. J. microsc. Sci. *57,* 301 – 304

Mulcrone R (2005) "Asteroidea" (On-line), Animal Diversity Web. http://animaldiversity.ummz.umich.edu/site/accounts/information/Asteroidea.html

Müller WEG, Zahn RK, Schmid K (1970) Morphologie und Funktion der Cuvierian Organe von Holothuria forskali Delle Chiaje (Echinodermata: Holothuroidea:Aspidochirota: Holothuriidae). *Z. Wiss. Zool.* 181: 219 – 231.

Müller WEG, Zahn RK, Schmid K (1972) The adhesive behaviour in Cuvierian tubules of *Holothuria forskali*: biochemical and biophysical investigations. *Cytobiol.* 5:335 – 351

Müller WEG, Hanske W, Maidhof A, Zahn RK (1973) Influence of apurinic acid on programmed synthesis in different in vitro systems. *Cancer research* 33, 2330 – 2337

Naldrett MJ (1993) The importance of sulphur cross-links and hydrophobic interactions in the polymerization of barnacle cement *J. mar. biol. Ass. U. K.* 73, 689 – 702

Naldrett MJ, Kaplan DL (1997) Characterization of barnacle (Balanus eburneus and B. crenatus) adhesive proteins. *Mar. Biol.* 127:629 – 635.

Nichols D (1959) The histology and activities of the tube feet of *Echinocyamus pusillus. Quart. J. Microssc. Sci.* 100:73 – 87

Nichols D (1960) The histology and activities of the tube feet of *Antedon bifida.* Q J *Microsc. Sci.* 101: 105 – 117

Nichols D (1961) A comparative histological study of the tube-feet of two regular echinoids. *Quart. J. Microsc. Sci.* 120:157 – 180

Nichols D (1966) Functional morphology of the water-vascular system Pp 219–240 in *Physiology of Echinodermata* , R.A. Boolootian , ed. Interscience Publishers, New York

Nicholson P, Shaw I (2000) Ancient Egyptian Materials and Technology p. 16 – 17 *Cambridge University Press*, Cambridge

Niederegger S, Gorb S, Jiao Y (2002) Contact behaviour of tenant setae in attachment pads of the blowfly *Calliphora vicina* (Diptera, Calliphoridae). *J. Comp. Physiol.* A187, 961 – 970

Ninan L, Monahan J, Stroshine RL, Wilker JJ, Shi R (2003) Adhesive strength of marine mussel extract on porcine skin. *Biomaterials* 24:4091 – 4099

Oakley BR, Kirsch DR, Morris NR (1980) A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* 105(2), 361 – 380

Okano K, Shimizu K, Satuito CG, Fusetani N (1996) Visualization of cement exocytosis in the cypris cement gland of the barnacle megabalanus *J. Exp. Biol.* 1996;199:2131 – 7

Ooka A, Garrell RL (2000) Surface-Enhanced Raman Spectroscopy of DOPA-containing peptides related to adhesive protein of marine mussel, *Mytilus edulis Biopolymers (Biospectroscopy),* Vol. 57, 92 – 102 (2000) John Wiley & Sons

Pearson WR, Morrow JF (1981) Discrete-length repeated sequences in eukaryotic genomes *Proc. Natl. Acad. Sci.* U S A. 1981 Jul;78(7):4016 – 20

Peppas NA, Langer R (1994) New challenges in biomaterials. *J. Exp. Zool.* 45:361 – 366

Peppas N, Sahlin J (1996) Hydrogels as mucoadhesive and bioadhesive materials: a review Elsevier Science Ltd *Biomaterials* 17 (1996) 1553 – 1561 Great Britain

Perpeet C, Jangoux M (1973) Contribution a l'etude des pieds et des ampoules ambulacraires d' *Asterias rubens* (Echinodermata, Asteroidea). *Forma and Functio* 6: 191 – 209

Picton (1993) A field guide to the shallow-water Echinoderms of the British Isles. Immel Publishing Ltd

Reichensperger A (1908) Die Driisengebilde der Ophiuren. *Z. Wiss. Zool.* 91:304 – 350

Rieger RM, Tyler S, Smith JPS, Reiger GE (1991) Platyhelminithes: Turbellaria . In: *Microscopic Anatomy of Invertebrates. Volume 3 Platyhelminthes and Nemertinea* (F. W. Harrison and B. J. Bogitsh, eds), pp 7 – 140. New York :Wiley-Liss

Rabilloud T (1990) Mechanisms of protein silver staining in polyacrylamide gels: a 10-year synthesis. *Electrophoresis*. 10, 785 – 94.

Rabilloud T (1999) Silver staining of 2-D electrophoresis gels. *Methods Mol. Biol.* 112, 297 – 305.

Santos R, Haesaerts D, Jangoux M, Flammang P (2005) The tube feet of sea urchins and sea stars contain functionally different mutable collagenous tissues. *The J. of Experimen. Biol.* 208, 2277 – 2288 Published by the Company of Biologists 2005

Schnurrer J, Lehr CM (1996) Mucoadhesive properities of mussel adhesive protein. *Inter. J. of Pharm.* 141, 251 – 256

Schütze J, Skorokhod A, Muller IM, Muller WEG (2001) Molecular evolution of metazoan extracellular matrix: cloning and expression of structural proteins from the demosponges *Suberites domuncula* and *Geodia cydonium. J. Molec. Evol.* 53:402 – 415

Semper C (1868) Die Holothurien. In: Reisen im Archipel der Philippinen Wissenschaftliche Resultate, 288 pp. Leipzig: W. Engelmann

Silverman H, Roberto FE (2007) Understanding mussel adhesion. *Mar. Biotechnol.* NY; 9(6): 661 – 681

Singer AJ, Thode HC (2004) A review of the literature on octylcyanoacrylate tissue adhesive. *Am. J. Surg.* 187:238 – 248

Smiley S (1994.) Holothuroidea. *In* F. W. Harrison and F.-S. Chia (eds.), *Microscopic anatomy of invertebrates,* Vol. 14, *Echinodermata,* pp. 401 – 471. Wiley-Liss, New York

Smith (1978) A functional classification of the coronal pores in echinoids. *Palaeontology* 21:759 – 789

Smith AB (1980) The structure, function, and evolution of tube feet and ambulacral pores in irregular echinoids. *Palaeontology* 23: 39 – 83

Smith AM, Callow JA, eds. (2006) Biological Adhesives. Springer, Berlin

Smith AB (2006) The pre-radial history of echinoderms *Geol. J. 40:*255 – 280

Smith JE (1937) The structure and function of the tube feet in certain echinoderms. *J. Mar. Biol. Ass U. K.* 22: 345 – 357

Smith JE (1947) The activities of the tube feet of *Asterias rubens* L.I. The mechanics of movement and of posture. *Quart. J. Microsc. Sci.* 88: 1 – 14

Southward AJ, Crisp DJ (1987) Barnacles CRC Press

Strathmann RR (1978) Larval settlement in echinoderms. In: Chia FS, Rice ME (eds) Settlement and metamorphosis of marine invertebrate larvae. Elsevier-North Holland, New York, pp 235 – 246

Strausberg Rl, Link RP (1990) Protein based medical adhesives . Trends Biotechnol 8 53-57 *Biol. Ecol*. 160: 205 – 220.

Switzer RC, Merril CR, Shifrin S (1979) A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. *Anal. Biochem.*. 98(1), 231 – 7.

Tamarin A, Lewis P, Askey J (1976) The structure and formation of the byssus attachment plaque in *Mytilus. J. of Morph.* 149, 199 – 222

Thomas L, Hermans C (1985) Adhesive interactions between the tube feet of a starfish *Leptasterias haxactis, and* substrata. *Biol. Bull.Mar. Biol. Lab.,* Woods Hole 169:675 – 688

Taylor SW, Waite JH (1997) Marine adhesives: from molecular dissection to application. In: Mc Grath K, Kaplan D (eds) Protein based materials. Birkhauser, Boston, pp 217 – 248

Tyler S (1988) The role of function in determination of homology and convergence-examples from invertebrate adhesive organs. In: *Fortschritte der Zoologie. Volume 36 Free-living and Symbiotic Plathelminthes (*P. Ax, U. Ehlers and B.Sopott-Ehlers, eds) pp.331 – 347.Stuttgart: Gustav Fisher Verlang

Tyler S (1976) Comparative ultrastructure of adhesive systems in the Turbellaria. *Zoomorphologie* 95, 1 – 15

Tyler S, Reiger GE (1980) Adhesive organs of the Gastroticha. I Duo-gland organs. *Zoomorphologie* 95 1 – 15

Tamarin A, Lewis P, Askey J (1976) The structure and formation of the byssal plaque forming region in *M. californianus. J.Morphol.*149:199 – 222

Tai TH, Tanksley SD (1990) A rapid and inexpensive method for isolation of total DNA from dehydrated plant tissue - *Plant Mol. Biol.* Reporter – Springer

Van Burik (1998) Comparison of six extraction techniques for isolation of DNA from filamentous fungi – *Med. Mycol.*, Blackwell Synergy

VandenSpiegel D, Jangoux M (1987) Cuvierian tubules of the holothuroid *Holothuria forskali* (Echinodermata): A morphofunctional study. *Mar. Biol.* 96:263 – 275.

VandenSpiegel D, Jangoux M (1993) Fine structure and behaviour of the so-called Cuvierian organs on the holothuroid genus *Actinopyga* (Echinodermata). *Ac. Zool.* (Stockh.) 74:43 – 50.

VandenSpiegel D, Flammang P, Fourmeau D, Jangoux M (1995) Fine structure of the dorsal papillae in the holothuroid *Holothuria forskali* (Echinodermata), *Tissue and Cell* 27: 457 – 465

VandenSpiegel D, Jangoux M, Flammang P (2000) Maintaining the line of defense: Regeneration of Cuvierian tubules in the holothuroid *Holothuria forskali* (Echinodermata). *Biol. Bull*. 198:34 – 49.

Wainwright SA, Biggs WD, Currey JD, Gosline JM (1976) Mechanical Design in Organisms. *Princeton University Press*, Princeton. 423 pp.

Waite JH (1982) Evidence for a Repeating 3, 4-Dihydroxyphenylalanine- and Hydroxyproline-containing Decapeptide in the Adhesive Protein of the Mussel, *Mytilus edulis* L. *The J. of Biol. Chem.* Vol. 258, No. 5, Issue of March 10, pp. 2911 – 2915, 1983

Waite JH (1983) Adhesion in bysally attached bivalves. *Biological Review of the Cambrigde Phil. Soc.* 58, 209 – 231

Waite JH (1986) Mussel glue from *Mytilus californianus* Conrad: a comparative study *J.comp. Physiol.* B 156, 491 – 496

Waite JH (1987) Nature's underwater adhesive specialist. *Int J adhesion Adhesive*7:914

Waite JH, Qin, X, Coyne JK (1998) The peculiar, collagens of mussel byssus Matrix. *Biology* vol. 17/1998 pp.93-106 Gustav Fischer Verlag

Waite JH, Qin X (2001) Polyphosphoprotein from the adhesive pads of Mytilus edulis. *Biochemistry* 40:2887 – 2893

Walker G (1987) Marine organism and their adhesion In: *Synthetic adhesives and* sealants (W. C. Wake ed) Critical Reports on Applied Chemistry, Volume 16, pp.112 – 135. Published for the Soc. of chem. Industr. Chichester: John Wiley and Sons

Walker G (1970) The histology, histochemistry and ultrastructure of the cement apparatus of three adult sessile barnacles, *Elminius modestus, Balanus balanoides* and *Balanus hameri. Mar. Biol.* 7, 239 – 248

Walker G (1971) A study of cement apparatus of the cypris larva of the barnacle *Balanus Balanoides Mar. Biol.* 1971; 9:205 – 12.

Walker G (1999) Cirripedia. In: F. W. Harrison and A. G. Humes (eds.), Microscopic Anatomy of Invertebrates 9: Crustacea, John Wiley, New York, Chichester, pp. 249 – 311.

Warren G, Gidmark D (2001) Canoe Paddles: A Complete Guide to Making Your Own p.80 Firefly Books Ltd, London

Weber K, Osborn M (1969) The reliability of molecular weight determinations by dodecyl sulfatepolyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406 – 4412

Weber DF, Shaw CF, Petering DH (1987) *Euglena gracilis* Cadmium binding Protein-I1 Contains Sulfide Ion. *The J. of Biol. Chem.Vol.* 262 No. 15 Issue of May 25 pp. 6962 – 6964

Welsch RE (1977) Stepwise multiple comparation procedures. *J. Am. Stat. Assoc*. 72, 566 – 575.

Whittington ID, Combes C (1994) Second International Symposium on Monogenea Montpellier and Sete, France. *Inter. J. for Parasitiol.* 24, 445 – 447

Wiegemann M, Burkard Watermann B (2004) The impact of desiccation on the adhesion of barnacles attached to non-stick coatings. *Biofouling* Volume 20, Issue 3 2004, pages 147 – 153

Wiegemann M (2005) Adhesion in blue mussels (*Mytilus edulis*) and barnacles (*Genus Balanus*): Mechanisms and technical applications *Aquat. Sci.* 67 (2005) 166 – 176

Williamson DI (2003) The origins of Larvae ,xviii+261 pp Kluwer Dordrecht

Young GA, Crisp DJ (1982) Marine animals and adhesion . In: Adhesion 6 (Allen , K. W.ed) *Applied Sci. Publishers, Ltd Essex , England*, pp.19 – 39

Zahn RK, Müller WEG, Michaelis M (1973) Sticking mechanisms in adhesive organ from a Holothuria. *Res. Mol. Biol.* 2:47 – 88.

10. List of abbreviations

APS	Ammonium persulfate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
ºC	degree centigrade
Ca	Calcium
Cd	Cadmium
cm	Centimetre
Cy3	indocarbocyanine 3
1, 2-D gel	one, two-dimensional gel electrophoresis
DAPI	4',6-diamidino-2-phenylindole
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOC	Deoxycholic acid
DOPA	3,4-dihydroxy-L-phenylalanine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay,
e.g.	exempli gratia (Latin), for example
Et al.	id est (Latin), that is
Fig	Figure
G	Gram
x g	times gravity (units of gravity)
GB	glass beads
HCl	hydrochloric acid
H&E	Haematoxylin and Eosin

HPLC	High-performance liquid chromatography (or High pressure liquid chromatography)
dH_2O	Distilled water
H	Hour
IgG	Immunglobuline G
K	Potassium
kDa	kilo-Dalton
L	Litre
M	molar (mol/l)
mA	milli-Amperes
MeOH	Methanol
Mefp	Mytilus edulis foot protein
Mg	Magnesium
$MgCl_2$	magnesium chloride
min	Minute
ml	millilitre
mm	Millimetre
mM	Millimolar
N	number of repetition
NaCl	Sodiumchloride
$NaHCO_3$	sodium hydrogen carbonate
NaH_2PO_4	sodium phosphate, monobasic
Na_2SO_4	Sodium Sulphate
NaOH	Sodium hydroxide
NBT	4-Nitroblautetrazoliumchlorid
ng	Nanogram
nm	Nanometre
N/m^2	Newton Per Square Meter

no	Number
NP-HPLC	Normal Phase- High-performance liquid chromatography
NP_{40}	nonyl phenoxylpolyethoxylethanol
*P	Significance
PAS	periodic acid
PAGE	Poly-Acrylamid-Gelelectrophoresis
PBS	phosphate buffered saline
PoAb	Polyclonal antibody
pH	potentia hydrogenii
PVDF	Polyvinylidene Fluoride
RP-HPLC	Reverse Phase- High-performance liquid chromatography
rpm	rounds per minute
SD	Standard deviation
SDS	sodium dodecyl sulfate
sec	second(s)
SEC	Size Exclusion Column
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline- Tween
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
TCEP	tris(2-carboxyethyl)phosphine
Tris	tris(hydroxymethyl)aminomethane
Tween 20	Polyoxyethylene (20) sorbitan monolaurate
TEM	Transmission Electron Microscope
UV	Ultraviolet
v/v	Volume/volume (Vol. %)
µl	Microlitre
µm	Micrometre