Freshwater Mussel Adhesive Protein Localization and Characterization

by

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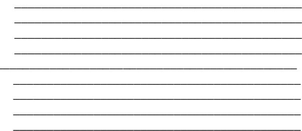
B.A.Sc. Thesis



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Abstract

Bioadhesives have presented their superiority in many clinical applications. However, current bioadhesives are mainly limited by the complexity caused by wet adhesion. Although dihydroxyphenylalanine (DOPA)-adhesives based on marine mussel model have been extensively investigated and advanced, they are not ideal in the clinical setting. Freshwater mussels, however, exhibit different underwater adhesion mechanism comparing to marine mussels. This is concluded primarily considering the distinct protein localization and composition of little DOPA ($< 1mol$) in the adhesive system of freshwater mussel. In this thesis, the underwater mechanism of invasive freshwater mussel *Dreissena bugensis* (quagga mussel) and *Dreissena polymorpha* (zebra mussel) found in the Great Lakes is investigated specifically. Through a buffer-based optimization on the extraction of zebra mussel foot protein, it was discovered phosphate buffer exhibited superior performance in collecting bulk plaque proteins, while sodium borate buffer presented greater efficiency in footprint protein collection. Furthermore, three staining-based approaches on the characterization of posttranslational modifications in quagga mussel protein were demonstrated. The results have promisingly indicated phosphorylation is presented in *D. bugensis* foot protein (Dbfp7) by approximately one phosphate per protein. It was further justified by the prediction results from Predictor of Naturally Disordered Regions(PONDRs) tool. On the other hand, the percentage phosphate presented a non-linear relationship with the concentration of Dbfp7, indicating phosphoylation does not occur for every Dbfp7. Through a coherent understanding of underwater adhesion mechanism utilized in freshwater mussel adhesive protein, this work provide fundamentals in the development and advancement of novel non-DOPA-dependent water resistant bioadhesives.

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Chapter 1

Introduction

1.1 Background

Bioadhesion refers to an interface phenomenon in which the adherence of natural or synthetic materials to biological surfaces occurs [1]. Mussel attachment to the underwater surface is a typical example of natural bioadhesion. This natural adhesion involves the secretion of byssus, a non-living anchor consisting of a bundle of proteinaceous filaments. Each filament adjoins the base of the animal's foot on one end, while terminating distally with a flattened plaque on the other end, which mediates sub-stream adhesion [2], [3]. Extensive study of this phenomenon has been investigated for marine mussels, where the amino acid dihydroxyphenylalanine (DOPA) predominates the adhesive function [4]. However, recent discovery arose different results in freshwater mussels [3], [5]. Freshwater mussels live in a different habitat which contains a significantly lower concentration of salt compared to where marine mussels reside. Accordingly, proteins that mediate the adhesion of freshwater mussels are also different from marine mussels [6], [7]. Through this section, we will gain a better understanding on current freshwater mussel adhesion mechanism and byssal protein knowledge, also investigating the well-established marine mussel model that have made significant contributions in the development of bioadhesives.

1.1.1 Freshwater Mussel Vs. Marine Mussel

Dreissena polymorpha (known as zebra mussel), and *Dreissena bugensis* (known as quagga mussel) are freshwater mussel invasive to the Great Lakes region in Ontario. Introduced in the 1980s, these species were accidentally carried from the cargo ships from Europe, and discharged into Lake St. Clair near Detriot, Michigan in North America. They were examined to be environmentally and financially costly due to their heavy adhesion in areas such as drinking water treatment and electric power generation facilities [8], [9]. Their success in colonizing the Great Lakes is partly due to the strength of their attachment to underwater substrates. This attachment is discovered to be based on a proteinaceous anchor called byssus [6]. Research on this attachment have already been relatively mature in terms of marine mussels, where their adhesion is primarily based on the amino acid DOPA [10]. This mechanism has been well understood and utilized in applications such as synthetic polymer adhesives. Freshwater mussels, however, have significantly lower DOPA levels than their marine counterparts, ranging from 0-1.2 moles DOPA in quagga mussel, and it is distributed equally

throughout the threads and plaques rather than localized at the interface [6]. In contrast, the marine mussels Mytilus edulis have adhesive proteins containing from 20-28 moles of DOPA [11]. This suggests that there must be another non-DOPA based mechanism of adhesion.

Figure 1.1 illustrates the difference in morphology and size between *Dreissena bugensis* (quagga mussel) and *Dreissena polymorpha* (zebra mussel). Although quagga mussels were introdcued several years later than zebra mussels, quagga mussels are outcompeteing zebra mussels in many sympatric areas due to their superior ability in tolerating lower oxygen concentrations [12], [13]. Furthermore, quagga mussels are able to colonize sediment, which surpass zebra mussels at low food concentrations and lower temperatures [14]. These advantages render quagga mussels a superior model in the study of freshwater mussel adhesive mechanism.

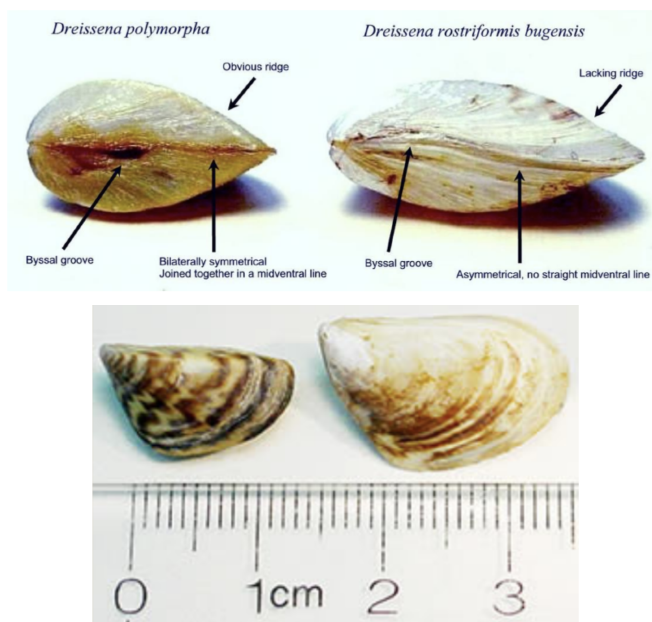


Figure 1.1: Comparison of quagga mussel and zebra mussel. Dorsal-ventral and lateral view of the zebra (left) and quagga mussels (right) with differences in morphology and size, image adapted from U.S. Geological Survey

1.1.2 Marine Mussel Adhesive Protein

Marine mussel foot proteins (Mfps) have been the centre of many studies in the development of bioadhesives due to their strong and flexible water-resistant adhesion, along with their biodegradability and biocompatibility over the last few decades. 25 marine mussel *mytilus* foot proteins have been identified with seven distinct types of Mfps (Mfp1 to Mfp7) demonstrating structural and functional competence in the adhesion mechanism. Among all the discovered proteins, DOPA — the posttranslational hydroxylation of tyrosine residue has been identified and discovered to be the most abundant among these candidates. The hydroxylation of tyrosine enhances the hydrogen bond between hydrophilic surfaces, also allowing proteins to interact and cross-link with each other by oxidative conversion to dopaquinone, a molecule that plays a crucial role in the strength of marine mussel underwater adhesion [15]. On the other hand, although the presence of DOPA has tradition-

ally been associated with strong cross-linking and covalent coupling, recent experiments discovered that uniformly high DOPA oxidation to cross-links indeed leads to high cohesive strength but interfacial failure, while low DOPA oxidation results in better adhesion at the expense of cohesion. These results imply that there is a balance between these two extremes in understanding fundamentals of marine mussel adhesion [16].

1.1.3 Freshwater Mussel Byssal Protein and Dbfp7

In order to investigate the adhesive mechanism of invasive freshwater mussel including quagga mussels and zebra mussels, we specifically look at their foot organ. The foot is primarily used for locomotion of mussel and secretion of the byssus. Specifically, the byssus is secreted by the glands inside this foot organ of the mussel, and therefore byssal proteins are referred to as "foot proteins" [6]. As shown in **Figure 1.2**, the byssus consists of a bundle of threads, attached to the body of the mussel by a stem. Each byssus consists of approximately 30-120 threads (20-50 um wide), and each thread is tipped with a sticky plaque [7]. At the bottom of each plaque, there exists a thin (approximately 10nm) electron dense layer, referred to as the "footprint" [17].

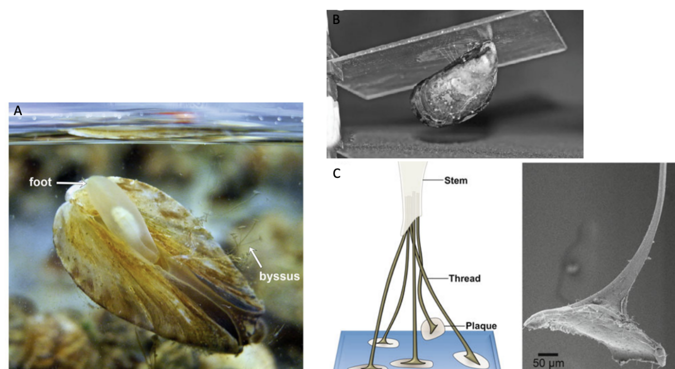


Figure 1.2: Freshwater mussel and the byssus. (A) The image of a freshwater mussel. (B) The image of a freshwater *Dreissena bugensis* attaching to the side of a glass aquarium. The byssus is visible, as is the foot of the mussel, which produces the byssus. (C) The byssus, consisting of several threads and plaques attached to a substratum, is shown schematically (left) and as a SEM micrograph of a detached plaque and thread, image adapted from Sone (2016).

The foot proteins of freshwater mussel have been identified ad characterized over time. To be more precise, previous works have successfully demonstrated and named novel proteins in both zebra mussel and quagga mussel [4], [7], [18], [19]. Specifically, quagga mussel *D. bugensis* foot proteins 0, 1, 2, 3 (Dbfp0-3) (**Figure 1.3**) and zebra mussel *D. polymorpha* foot proteins 1, 2, 3 (Dpfp1-3) (**Figure 1.4**) were identified to be homologous to each other[6], [17]. Note that in **Figure 1.4**, Dpfp3 is not visible in the gel, this is because this protein has been renamed Dpfp7 [20].

However, in the initial stage of quagga mussel foot protein identification, the quagga mussel proteins were only isolated post-secretion with a DOPA-specific stain, which limited the identification of DOPA-containing byssal proteins only [17]. To circumvent the only-DOPA containing proteins identification, subsequent studies used Potassium chloride (KCL) injection further identified 8 novel byssal proteins [4]. Gel bands were being analyzed that the majority of the adhesive protein candidates localized in the 6/7 kDa bands, implying the protein composition in these gel bands may

reveal the primary adhesive protein candidates. Byssal protein Dbfp7 was identified to be the most abundant in the whole thread-plaque extract (previously identified as Dbfp3). Dbfp7 was also discovered to be rich in the 6/7 kDa bands. These discoveries render it a great contributor in the freshwater mussel adhesive mechanism and the protein of interest for many studies.

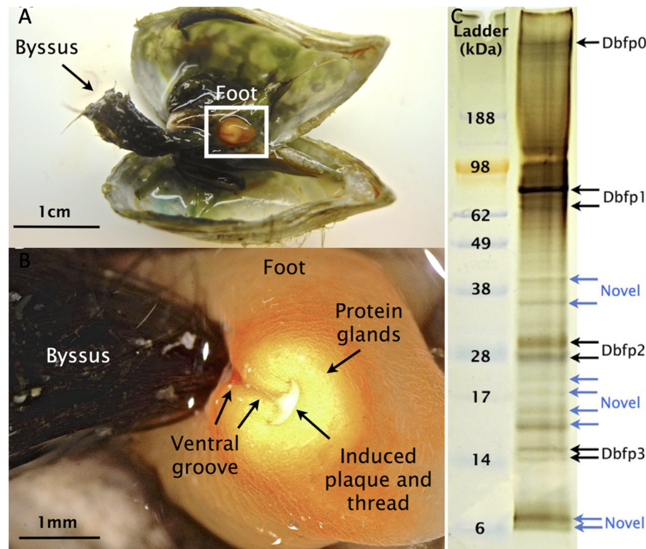
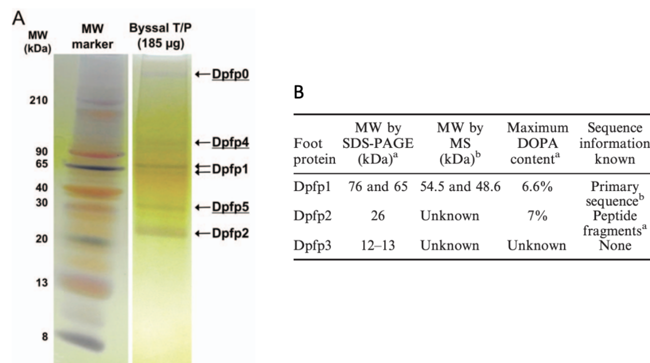


Figure 1.3: Initial studies of qugga mussel foot and foot protein. (A) Adult quagga mussel flayed open exposing the foot organ responsible for byssal secretion(left). (B) Quagga mussel interior(right). (C) SDS-PAGE gel visualized with SilverQuest silver stain, left lane contain standard ladder and right lane contain 15 gel bands. Image adapted from Rees et al. (2016).



Foot protein	MW by SDS-PAGE (kDa)[a]	MW by MS (kDa)[b]	Maximum DOPA content[a]	Sequence information known
Dpfp1	76 and 65	54.5 and 48.6	6.6%	Primary sequence[b]
Dpfp2	26	Unknown	7%	Peptide fragments[a]
Dpfp3	12–13	Unknown	Unknown	None

Figure 1.4: Initial studies of zebra mussel byssal protein. (A) Byssal proteins identified in an extract from complete byssal threads and plaques (T/P). (B) Summary of information on the three known D.polymorpha foot proteins (Dpfp 1-3). Image adapted from Gantayet (2013).

In the case of zebra mussel, it began with the initial discovery of 3 DOPA containing proteins (Dpfp 1-3) [6], and proceeded by the creation of a cDNA library of the *Dreissena polymorpha* foot transcriptome [21], which allowed for the recent discovery of ten novel functional byssal proteins [3]. However, it was not until the identification of the zebra mussel *Dreissena polymorpha* genome in 2019 that the full proteome could be found [19].

On the other hand, the initially discovered Dpfps and Dbfps 1,2,3 are DOPA-containing proteins only. In recent studies in the Sone lab proteome, it was discovered a list of novel Dpfps and Dbfps that are involved in byssal adhesion **Figure 1.5**. However, localization is required to identify the specific proteins that are involved in interfacial adhesion as opposed to thread formation [22].

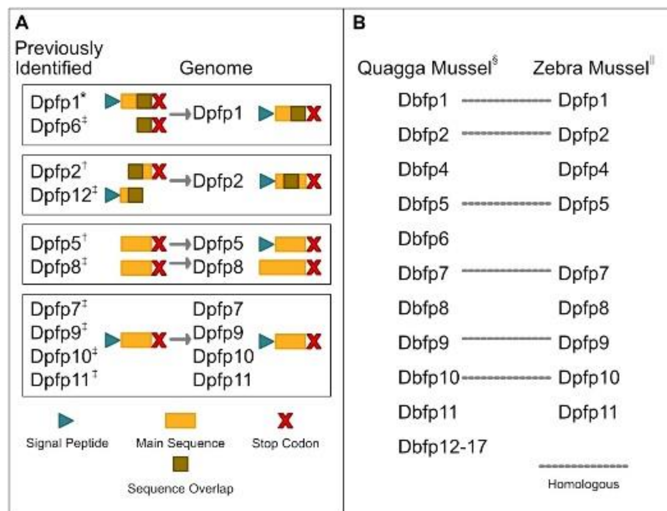


Figure 1.5: New discoveries of zebra mussel and quagga mussel byssal protein. (A) Summary of the re analysis of the Dpfp proteome using the genome assembly. Proteins on the left were sequenced previously and proteins on the right represent full length Dpfp protein sequences identified in the genome. (B) Homology between the quagga mussel byssal proteome and the zebra mussel byssal proteome. Image adapted from Obille (2022).

1.2 Rationale and Motivation

1.2.1 Limitation of Current Bioadhesives

Polymeric materials that act as adhesives, are widely used in the design of synthetic materials that adhere to biological tissue. They have superiority in clinical applications as tissue adhesives, hemostats, and tissue sealants. However, the current bioadhesives are mainly limited by adhesion in aqueous environments, imposing considerable challenges in a variety of applications including medical, dental and surgical applications. In the presence of moisture, most synthetic adhesives suffer detachment and deterioration [23]. For example, the use of surgical glues serves a crucial role in the success of minimally invasive surgeries. This mechanism particularly relies on the attachment of objects to tissue without penetration of compression. The standard method of fastening tissue involves mechanical methods such as staples or sutures. However, these practices can impose critical surgical failures and long-term scar if the surgical conditions become moist and turbulent [24]. Another typical example is the irreversible loss of tooth during the replacement of dental restorations after years due to weakened bonding [25]. These problems have led to the demand of bioadhesives that are nature-inspired and non-toxic, also capable of robust and flexible adhesion in wet environment.

In search of a bioadhesive that match within the design criteria as well overcoming the challenges presented by current synthetic adhesives, marine mussel foot protein and its DOPA characteristic

has become a well-established role model for investigating biomimetic wet adhesion over the last few decades. Although DOPA-adhesives have been extensively investigated and advanced, they are not ideal in the clinical setting. There are three main challenges laid under this problem: (1) Insufficiency and impurity of isolated native Dbfp7 proteins [26]. (2) Effectiveness of recombinantly produced marine mussel adhesive proteins is limited to the action of tyrosinase that can inhibit the DOPA effect (hydroxylation of tyrosine residues) [27]. (3) Instability and cytotoxicity characteristics of Dbfp7 protein [28].

On the other hand, freshwater mussels and marine mussels share a similar foot structure that mediates surface adhesion — known as the proteinacious anchor byssus extending from the foot of the mussel by threads and terminating by plaques that are in contact with the adhesion surface. Marine mussel adhesion has been well studied to be directly associated with the presence of DOPA structure. In comparison, the adhesion mechanism of freshwater mussel remains largely a mystery where only trace of DOPA was discovered in the byssal protein, the question regarding to the non-DOPA adhesion mechanism was raised and remained unanswered.

1.2.2 Significance of the Study

The primary motivation of the thesis research centred around the unanswered questions in freshwater mussel adhesion including the localization of proteins responsible for freshwater mussel underwater adhesion and the non-DOPA-dependent mechanism of adhesion. Through a coherent understanding of freshwater mussel adhesive protein localization and their chemical nature, this research provides a basis for the development of anti-fouling surfaces against freshwater mussels, which can help control biofouling of invasive species in fresh water habitats.

Furthermore, this work can be further utilized for the development of bioadhesives. Since it is very difficult to create biocompatible adhesives that work perfectly in aqueous environments, gaining new insight into the means of freshwater mussel adhesion can provide a basis for the development of bioadhesives without being limited by DOPA chemistry. Freshwater mussel is a well-established role model in studying underwater adhesion, as well as the advancement of novel non-DOPA-dependent water resistant bioadhesives such as self-healing materials for wound closure and advanced coatings in aqueous environment.

1.3 Project Objectives and Goals

This research consists of two independent sections: (1) Optimization of Zebra Mussel Byssal Protein Extraction. (2) Analysis of Posttranslational Modification of Quagga Mussel Protein, each contributing to a broader understanding of the freshwater bioadhesion.

1.3.1 Objective 1: Optimization of Zebra Mussel Byssal Protein Extraction

Although attempts have been made in previous studies to extract byssal proteins of zebra mussel, the collected amount of protein was no where near sufficient for sophisticated analysis. Consequently, the localization of proteins within the zebra mussel byssus has not yet been identified successfully at the current state of the art. In order to fill this research gap, the first objective of this study is to

investigate the alternatives of extraction buffer that potentially optimizes the extraction process. By selecting the optimal buffer that extracts the most proteins on an efficient basis, sufficient proteins ($> 1ug$ of each group)could be collected for amino acid analysis and differentiated as belonging primarily to the bulk plaque, thread, or footprint. This allows us to examine candidats' adhesive characteristics as they are likely to be enriched at and around the interface.

The completion of this work can be potentially utilized to establish a coherent understanding of zebra mussel protein localization within the byssus, similar to the work completed for quagga mussels that led to the identification of Dbfp7 as the protein that potentially dominates in adhesive mechanism [19].

1.3.2 Objective 2: Analysis of Posttranslational Modification of Quagga Mussel Byssal Protein

Posttranslational modifications (PTMs) refers to the reversible or irreversible chemical changes proteins may undergo after translation. Although DNA typically encodes 20 primary amino acids, proteins contain more than 140 different residues because of PTMs (**Figure 1.6**)[29]. It exhibits a crucial role in diversifying proteome and allows for tailoring of both the structure and function of proteins within cells.

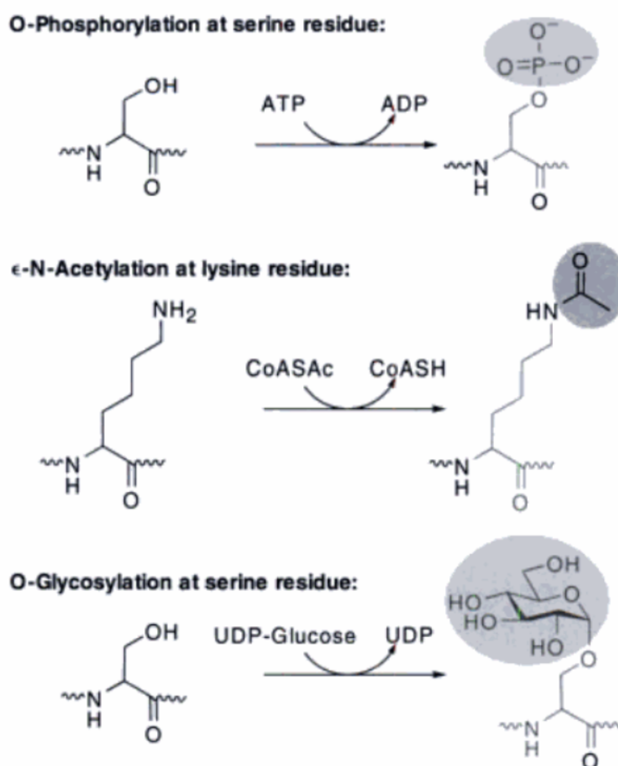


Figure 1.6: Posttranslational modifications of proteins (PTMs). Covalent addition of a substrate fragment to a protein side chain catalyzed by a posttranslational modification enzyme. Examples shown are phosphorylation, acetylations, and glycosylations. Image adapted from Walsh (2006)

Despite their theoretical ubiquity in the potential of post-translational modifications in synthetic

biomaterials has remained largely a mystery. A proof of concept study has demonstrated feasibility of a genetically encoded biohybrid material through posttranslational modification functionalized with a C14 alkyl chain. After such modification, it can exhibit a temperature-triggered hierarchical self-assembly [30]. In addition, DOPA's contribution to mussel adhesion mechanism is mainly due to PTM - hydroxylation into tyrosine residue.

In order to understand other PTMs that assist in freshwater mussel adhesion mechanism, as well developing synthetic bioadhesives that exhibit multiple characteristics, the goal of this part of the research is to characterize PTMs on freshwater mussel adhesive proteins. Specifically, the objective is to identify and quantify the extent of such modifications in the byssal proteins of quagga mussel.

Chapter 2

Optimization of Zebra Mussel Byssal Protein Extraction

2.1 Literature Review

According to research on existing studies and discussions with Mimi Simmons and Professor Matthew Harrington at McGill University, we identified three candidates of extraction buffers used for zebra mussel protein extraction:

1. Sodium Borate buffer (SB): Containing 0.2M Sodium Borate, 4M Urea, 1mM Potassium cyanide (KCN), 1mM Ethylenediaminetetraacetic acid (EDTA), 10mM Ascorbic Acid (pH 8) [6].

2. Phosphate buffer (PH): 20mM Phosphate buffer (pH 7), 4M Urea, 1mM Potassium cyanide (KCN), 1mM Ethylenediaminetetraacetic acid (EDTA), 10mM Ascorbic Acid (pH 8) [31].

3. AcH buffer (AcH): Containing 5% Acetic acid, 8M Urea [6].

A pretreatment of 1% acetic/N-phenylthiourea were also examined following discussion with Mimi Simmons and Professor Matthew Harrington [31].

2.2 Method

The experimental method involves extraction buffer preparation and protein extraction from naturally secreted byssus of zebra mussels, with distinct tubes collecting byssal proteins from bulk plaque, thread, and footprint respectively.

Specificallty, with the aim of collecting naturally secreted bysuss, 25 mussels were positioned on the microscopic glass slide one day prior to extraction. On the second day, each mussel was examined on whether it has successfully laid byssus by gentling pulling on the mussel from the glass slide. Active mussels were isolated and transferred back into a separate water tank for further experimentation.

Byssus were examined under the microscope and detached from mussel body through use of a scalpel, and then threads were separated from plaques via a scalpel. Plaques were scraped off the glass slide with a single pass by a (0.009" double edge) razor blade, held at approximately 45 degrees from vertical. Footprints were circled with hydrophobic barrier pen and left on glass slides.

Extraction buffers were prepared in advanced of the experiment and 250ml of each was stored in an apparatus that used to samples (bulk plaques and threads) mechanically by hand. In terms of the footprint, 10 uL extraction buffer was allowed to sit on footprints for 6 minutes, and then collected. Samples were also probe sonicated at 20%, for 40 seconds for further homogenization. A total of more than 24 byssus from the successful candidates were collected for each extraction buffer containing 12 samples (**Figure 2.1**).

On the second day, centrifugal filtration procedure and bis (2-hydroxyethyl)iminotris (hydroxymethyl)methane (Bis-Tris) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were performed following the extraction of proteins for protein concentration and separation. The gel was then being immersed in the fixing solution overnight in preparation of total protein staining. On the third day, silver staining was performed using Sigma-Aldrich ProteoSilver Stain [32]. Images were obtained for the successfully stained gel. The absorbance of the collected protein samples were also measured through nanodrop for analysis of protein concentration.

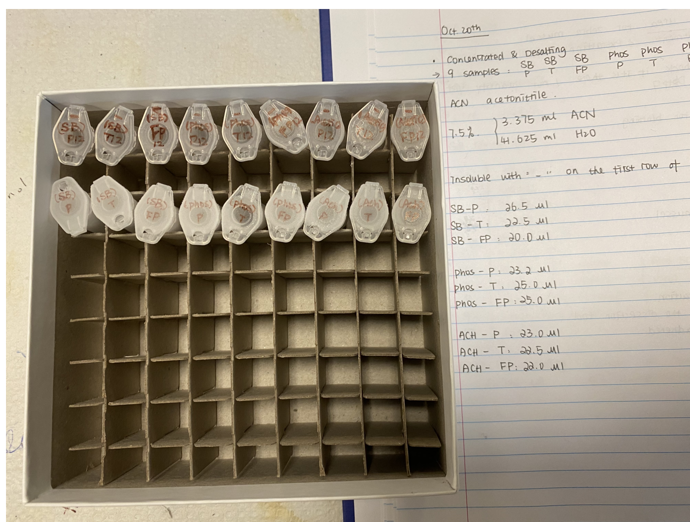


Figure 2.1: Sample collection of zebra mussel byssal protein. 1.5ml Eppendorf tubes with protein samples collected

2.3 Results and Discussion

This section provides a thorough demonstration of the experimental results and analysis on the performance of the selected buffers for freshwater mussel protein extraction.

2.3.1 Buffer Selection of Quagga Mussel Byssal Protein

The first set of the experiment was completed with quagga mussel due to the unavailability of zebra mussel in the laboratory. As both of the zebra mussels and quagga mussels are categorized into invasive freshwater mussels in the Great Lake, it is hypothesized that the performance of the extraction buffers may have some extent of similarities.

Only bulk plaques of quagga mussel were collected for this experiment. From the image of the silver-stained gel(**Figure 2.2**), proteins extracted in phosphate buffer (PH) are distinct comparing

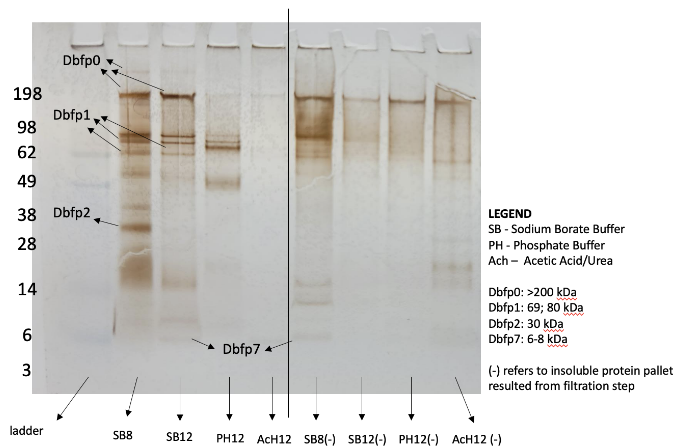


Figure 2.2: Silver-stained gel image with quagga mussel byssal protein

to proteins dissolved in sodium borate buffer (SB) according to the different protein bands shown on the gel. Specifically, Dbfp0, Dbfp2 and Dbfp7 are presented in SB but not in the PH, whereas protein band at 49 kDa in samples dissolved in PH does not appear in sample dissolved in SB.

The micromolar concentration of each sample was calculated from nanodrop absorbance using Beer–Lambert law given by

$$A = \epsilon c l,$$

where A is absorbance, ϵ is molar absorption coefficient, c is molar concentration, l is the optical path length (here ϵ is calculated from previous experimental data, $l = 1cm$)

Figure 2.3 demonstrate the protein concentration calculated from absorbance data (**Figure A.1**). It can be observed that there is a discernible increase in protein concentration of sample containing 12 bulk plaques comparing to sample containing 8 in SB, indicating a linear relationship between protein concentration and amount of bulk plaque. In addition, PH containing the same amount of 12 bulk plaque exhibited a slightly higher protein concentration. AcH buffer (AcH) does not improve extraction efficiency at all with only little protein collected. On the other hand, as the candidates being tested in this trial were not zebra mussel but quagga mussel instead, it was indispensable to continue conducting experiments using AcH on zebra mussels to examine any different effects.

2.3.2 Buffer Selection of Zebra Mussel Byssal Proteins

Similar experimental procedures have been followed with zebra mussel candidates. The zebra mussels were collected from Round Lake, Ontario. For the purpose of examining protein localization on byssus including bulk plaque, thread and footprint, proteins from different sections were collected separately in three protein low-bind tubes for each buffer. A silver-stained gel image and nanodrop results were obtained(**Figure 2.4; Figure 2.5**). Protein concentrations were calculated from the nanodrop absorbance (**Figure A.2**).

As shown in **Figure 2.4**, the silver-stained gel only exhibit vague protein bands. This phenomenon may be caused by the small-sized proteins resulted from small-sized zebra mussel collected (¡1cm). In addition, the exact number of plaque and thread count were not accurate according to the

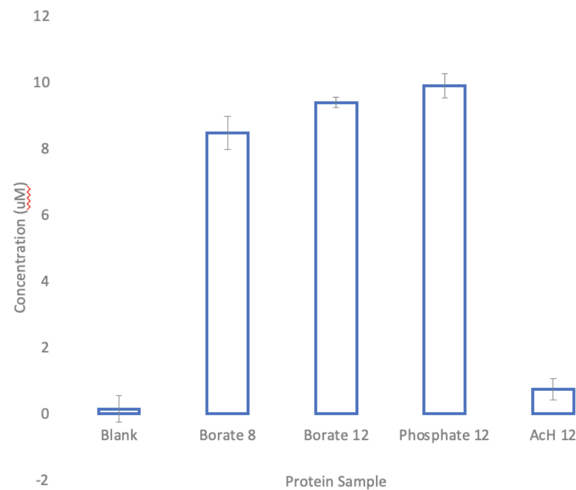


Figure 2.3: Micromolar concentration of quagga mussel byssal protein obtained from bulk plaque in different extraction buffers

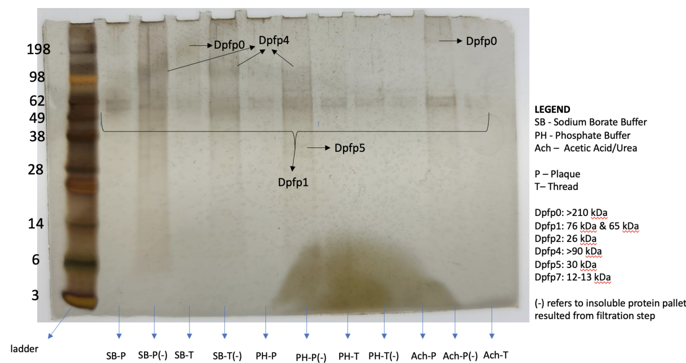


Figure 2.4: Silver-stained gel image with zebra mussel byssal protein from bulk plaque, thread and footprint **Zebra mussel used were collected from Round Lake, Ontario.*

same issue. On the other hand, zebra mussel byssal protein Dpfp0, Dpfp1, Dpfp4 and Dpfp5 can be potentially identified on gel[3], [6]. On the other hand, the band at around 49 kDa across all samples on the gel might be a keratin contamination exposed to the gel. From the protein concentration bar graph shown as **Figure 2.5**, PH results in a significant high protein concentration from bulk plaque in comparison to proteins extracted from threads and footprints. It also presents the highest performance in protein extraction comparing to other extraction buffers.

Similar experimental procedures have been performed for the second set of experiment with freshly collected zebra mussels from a different location Port Harbour, Mississauga in order to account for the low concentration of protein collected from previous trial. 19 zebra mussel candidates that were approximately 3-4 times larger than the ones used in the previous trial have been identified from a mixture of freshwater mussels collected. For the purpose of examining protein localization, proteins from bulk plaque, thread and footprint were collected separately in three protein low-bind tubes for each buffer.

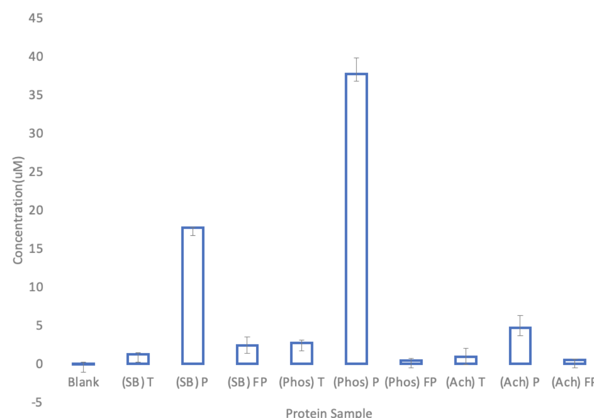


Figure 2.5: Micromolar concentration of zebra mussel byssal protein obtained from bulk plaque, thread and footprint in different extraction buffers **Zebra mussels collected from Round Lake, Ontario.*

Unfortunately, the silver-stained gel image obtained exhibited abnormal band patterns due to technical gel-related issues, which can not be evaluated for further analysis (**Figure A.1**). This rendered the third trial was not as successful as expected. On the other hand, the nanodrop results could still be analysed. As shown in **Figure 2.6** that present the protein concentration calculated from absorbance measured through nanodrop (**Figure A.3**), the large gap identified on the bar graph have demonstrated similar results to that of last experiment with small-sized zebra mussel candidates. Specifically, PH successfully extracted a significantly high protein concentration from the bulk plaque, whereas SB collected more proteins in the footprint. In addition, along with the size change of the zebra mussels used, the results have presented an overall higher concentration of the proteins collected in comparison of previous experiment.

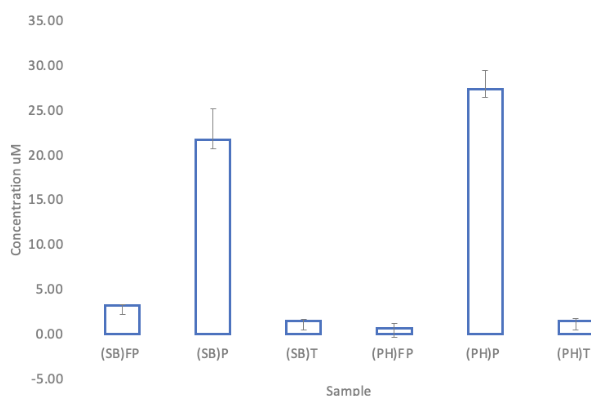


Figure 2.6: Micromolar concentration of zebra mussel protein obtained from bulk plaque, thread and footprint in different extraction buffer **Zebra mussels collected from Port Harbour, Mississauga.*

In addition, we aimed to further explore the effect of pretreatment in addition to the three extraction buffers used in previous experiment, where protein samples were pre-treated with 1%

acetic/N-phenylthiourea before storing them in the designated extraction buffers. Unfortunately, the experiment was forced to pause due to the delay of filtering tubes required for the centrifugal and filtration step. Therefore, no results was obtained for this experiment. The collected proteins were stored in the -20 Celsius degree freezer for future experiments.

Overall, the experimental data presented so far have indicated promising conclusions in terms of extraction buffer selection. Phosphate buffer (PH) and sodium borate buffer (SB) have presented similar results in terms of protein concentration, where phosphate buffer showed slightly higher efficiency in extracting proteins from the bulk plaque, while sodium borate buffer showed higher efficiency in extracting from the footprint. On the other hand, according to the low protein concentration obtained using AcH buffer, it indicated this is not feasible in the extraction of zebra mussel proteins.

2.4 Limitation and Future Study

Due to the uncertainties regarding to the shipment of experimental materials and the time constraints of this research project, the main focus of this research has centred more on the second objective. [1].

The next step in the optimization of zebra mussel byssal protein extraction is to complete the experiment by performing pretreatment with 1% acetic/N-phenylthiourea before dissolving into specific extraction buffers and to observe any effect on the extraction efficiency.

After determining upon the buffer that exhibits the highest extraction capacity, the ultimate goal is to understand the localization of the zebra mussel adhesive proteins by generating a volcano graph similar to what have been done for quagga mussel proteins in recent studies [22]. To achieve this goal, an amount of 1 ug of protein samples need to be collected and sent for further sophisticated analysis including amino acid analysis, liquid chromatography (LC) and mass spectrometry (MS) for a better demonstration of different adhesive protein identification and enrichment in each section of the byssus.

[1] Filtering tubes were ordered from different brands, experiment can be furthered upon the arrival of the tubes

Chapter 3

Analysis of Posttranslational Modification of Quagga Mussel Byssal Protein

3.1 Literature Review

Post-translational modification (PTMs) refers to the enzymatic modification of proteins following protein biosynthesis, which exhibits a crucial role in diversifying protein structures and functions.

Previous studies have shown that three types of PTMs, hydroxylation, glycosylation and phosphorylation, are presented in mussel adhesive proteins. Specifically, in Rzepecki's paper published in 1993, they discovered the two types of zebra mussel adhesive proteins Dpfp-1 and Dpfp-2 presenting extensive O-glycosylation on threonine and serine [6]. There are also other adhesive proteins such as Pvfp1 in marine mussels and cp-52k in barnacles, have shown evidence of glycosylation [1]. In addition, phosphorylation, another type of PTMs has been detected on serine in some marine adhesive proteins [33].

Based on existing studies, this research has been centred around detection and analysis of phosphorylation and glycosylation within the adhesive system of quagga mussel specifically, in order to investigate non-DOPA-based adhesion mechanism. Protein phosphorylation is one of the most common and important PTMs. It is a reversible mechanism consisting of the addition of a phosphate group (PO4) to the polar group R of various amino acids [34]. Consequently, this structure alteration modifies protein from hydrophobic apolar to hydrophilic polar, allowing protein to change conformation and bind molecules when interacting with other molecules. Glycosylation, another common PTM, involves the covalent attachment of several different types of glycans (also named carbohydrates, saccharides, or sugars) to a protein. The glycan chains not only contribute to structural and modulatory properties of proteins, also play an important role in recognition by other molecules, endogenous receptors, and exogenous agents [35]. Due to the fact that a large number of naturally occurring sugars can be combined to create a variety of unique glycan structures, as well as multiple enzymatic preference sites that further create diversity in where and how these sugars are linked to each other, studies related to detection of protein glycosylation can be challenging [36].

Therefore, this research will have a greater focus on protein phosphorylation over glycosylation.

Multiple approaches were utilized in previous studies that identified and characterized PTMs. In terms of specific phosphorylation detection protocols, Phosphate Assay and Pro-Q Phosphoprotein Gel Stain were utilized for the illustration of phosphorylation on specific proteins residues [37], [38]. As for glycosylation, existing studies have demonstrated feasibility of staining methods such as stains-all, periodic acid-Schiff (PAS) and affinity-based staining using lectins [18], [33], [39]. Further sophisticated analysis can be achieved through LC/MS following the staining methods.

3.2 Method

The experimental procedure generally consists of three sections: sample preparation, sodium dodecyl-sulfate polyacrylamide gel electrophoresis(SDS-PAGE) and PTMs detection.

Adhesive protein samples used in this research were quagga mussel proteins S1, S2 and Dbfp7. In sample preparation, quagga mussel byssal proteins were extracted from the phenol glands and dissolved into S1 and S2 buffer, where S2 refers to pellets taken from S1 sample dissolved in 5% acetic acid and 8M urea [40]. They are identified as byssal proteins which we are interested in. After sample preparation, the samples were going through the procedure of SDS-PAGE that electrically separate proteins due to their molecular weight. Dbfp7 were separated from S2 protein mixture and purified by high-performance liquid chromatography(HPLC). In this research, multiple staining-based approaches including stains-all, Pro-Q Phosphoprotein Gel Stain and Phosphate Assay were sequentially performed for phosphorylation detection and quantification based on literal review and experimental results along the way. Detailed staining methods are described in the section below.

3.2.1 Stains-all

Stains-all is a cationic carbocyanine multipurpose dye, it stains highly anionic protein blue, less anionic proteins pink, and glycoproteins purple. Due to the fact that phosphate is an anion, blue color could in part explain that these proteins are phosphorylated [41]. As a result, it allows us to observe directly from the gel whether any PTM occurs in protein samples. It also functions as an indicator for both phosphorylation and glycosylation according to different colors of the protein bands.

Here we followed an improved stains-all methods (ISA) which provides higher efficiency than the original method and could be completed within a time period of 60 minutes. After SDS-PAGE, gels were fixed in 30% v/v formamide for 2x20 minutes. Once completed, the solution was discarded and re-immersed in ISA working solution which contains 0.01% SA, 45 mM Tris-HCl (pH 9.2), 30% formamide for another 20 minutes. Finally, the gels were developed in developing solution containing 1% EDTA-2Na and 30% v/v EtOH for 1 minute. For each step, a tenfold excess of the gel volume was used with continuous gentle agitation of the plastic container [42].

3.2.2 Pro-Q Phosphoprotein Gel Stain

Pro-Q Phosphoprotein Gel Stain provides a method for selectively staining phosphoproteins in polyacrylamide gels, and ideal for phosphoproteomic studies and identification of kinase target in signal transduction pathways.

This proprietary fluorescent stain allows direct in-gel detection of phosphate groups attached to tyrosine, serine, or threonine residues in qugga mussel proteins without antibodies or radioisotopes comparing to complex detection methods. It can be completed in 5 hours excluding overnight fixing. In addition, Pro-Q stain is compatible with MS for further sophisticated analysis of the phosphorylation state of entire proteomes. The detailed experiment procedure in this study follows the staining protocol from Invirtrogen Pro-Q Diamond Phosphoprotein Gel Stain post SDS-PAGE [43].

3.2.3 Phosphate Assay

Phosphate Assay utilizes a proprietary formulation of malachite green and ammonium molybdate which forms a chromogenic complex with phosphate ion giving an intense absorption band around OD = 650nm [38]. As the post-experimental analysis requires known protein concentration, the mixture of protein samples S1 and S2 is not feasible. Therefore, purified Dbfp7 samples was used in this experiment instead.

Prior to the experiment, purified Dbfp7 were prepared following HPLC purification protocol[44]. Phosphate standard were prepared according to ratios listed in the Phosphate Assay protocol [38]. Duplicate samples were being loaded into a 96-well plate. 30 minutes after the phosphate reagent being added into each well, the plate was transferred to a plate reader for absorbance reading under 0D650nm. The entire process was done protected from light.

3.3 Results and Discussion

This section provides a thorough demonstration of the experimental results and analysis completed on PTMs of quagga mussel byssal protein S1, S2 and purified Dbfp7.

3.3.1 Phosphorylation and Glycosylation of Quagga Mussel S1 and S2 Protein Mixture

Considering the comprehensiveness and complication of this part of the research, a comprehending picture was first illustrated through the utilization of a relative general staining method Stains-all.

Two trials associated with Stains-all stain of SDS-PAGE on quagga mussel S1 and S2 protein samples were performed along with Coomassie blue stain and Silver staining as positive control. **Figure A.2** presents the gel image of the first trial. Although there are evident bands shown in the Coomassie-stained gel, only the ladder and a slight band shown around 6/7 kDa, where Dbfp7 residues could be recognized. In addition, protein bands are too faint to be color-differentiated. Possible factors could be the S1 and S2 proteins used were not freshly extracted, but collected 3 months ago and stored in the freezer. Furthermore, during the staining procedure, the stained gel was very sensitive to light, which faded immediately after taking out from the developing solution to the light box before imaging. Considering these potential issues, another set of experiment was performed. **Figure 3.1** demonstrates the the Stains-all-stained image along with a Coomassie stained-gel as positive control.

Freshly extracted S1 and S2 proteins were prepared in advance of the experiment. As observed in **Figure 3.1**, the gel image presents a variety of colored protein bands. Specifically, it can be

observed in the stains-all gel that a variety of colored-stained protein bands are presented. The byssal protein Dbfp7, as well as bands at 14, 38 kDa, stains blue on the stain-all gel, indicating these proteins are high anionic, and likely being phosphorylated. Specifically looking at the amino acid sequence of Dbfp7, the acidic variants have charge -1 to 0 and the basic variants have charge +1 to +4. This points to the possibility that Dbfp7 is phosphorylated, which would increase the negative charge of the proteins [22].

It can also be observed a band at 198 kDa, identified as byssal protein Dbpf0, stains purple, indicating their glycosylation characteristics. The pink bands can be recognized at 40 and 49 kDa, but they remain to be unidentified quagga mussel proteins based on protein sequence in previous studies.

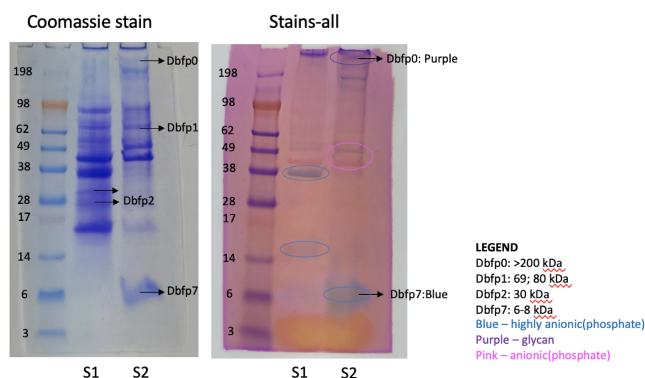


Figure 3.1: Stains-all gel image with quagga mussel protein S1 and S2 (Left: Coomassie stain; Right: Stains-all).

Following the promising results obtained from Stains-all, we continued to investigate phosphorylation in quagga mussel byssal proteins through the use of Pro-Q Phosphoprotein Gel Stain. Two trials associated with Pro-Q Phosphoprotein Gel Stain were performed along with Coomassie blue stain as positive control. In the first trial **Figure A.3**, the phosphate standard bands at 14 kDa stains on Coomassie but does not stain on the Pro-Q stain similar to Dbfp7 bands at 6/7 kDa. Potential rational can be that the gel was not imaged immediately after destaining but instead being immersed in the fixing solution overnight due to technical issue. Another reason could be that Dbfp7 has little phosphate. On the other hand, it can be concluded that there is no obvious difference in terms of protein bands comparing fresh S1 and S2 samples with old S1 and S2 samples (lane 2,3 vs lane 4,5 from **Figure A.3**). Another set of experiment was performed in the following week, where the gel was imaged immediately after destaining as instructed in the protocol.

Figure 3.2 demonstrates the the Pro-Q-stained gel image in the second trial along with a Coomassie stain as positive control, and Stains-all results obtained from previous experiment.

Specifically, it could be observed that Dbfp0, Dbfp1, Dbfp2 and Dbfp7 were recognized[19], but most are not visible on the Pro-q stained gel. Despite clear bands recognized at 38 and 42 kDa in S1, which stain blue and pink respectively on the stains-all gel, indicating they are potentially phosphorylated. Their presence on the Pro-q stained gels further justifies this conclusion. In addition, pink bands can be identified at 38, 42 kDa in S2 on the stains-all gel, that are also presented on Pro-q-stained gel. Although these proteins remain to be unidentified quagga mussel proteins based on protein sequence in previous studies. On the other hand, the stain seems to not stain for lower

molecular weight proteins, as exhibited by the lack of 14 and 18 kDa phosphoprotein standard bands on the Pro-Q-stained gel.

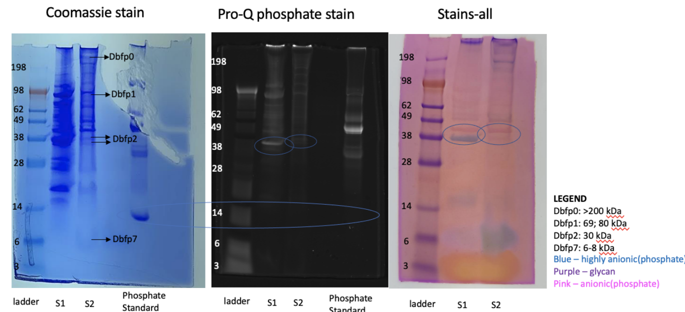


Figure 3.2: Pro-Q Phosphoprotein gel image with quagga mussel protein S1 and S2 (Left: Coomassie stain; Centre: Pro-Q Phosphoprotein stain Right: Stains-all).

3.3.2 Phosphorylation of Quagga Mussel Protein Dbfp7

To quantitatively analyze phosphorylation in quagga mussel byssal proteins, further investigation through the use of Phosphate Assay was performed. The obtained absorbance was presented in **Figure 3.3; Figure A.4**).

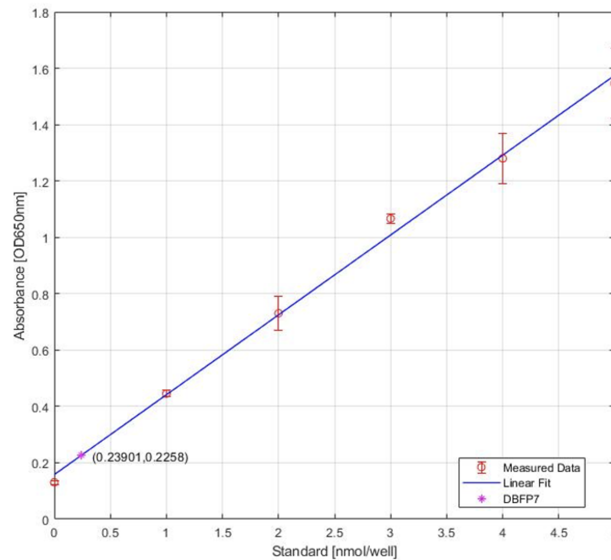


Figure 3.3: Trial 1 Phosphate absorbance of Dbfp7. Line of best fit generated from standard curve with absorbance(OD650nm) vs. phosphate concentration (nmol/well).

From the line of best fit ($y = 0.2834x + 0.1581$ $R^2 = 0.9997$) generated from the phosphate standard absorbance, we obtained the phosphate level of the Dbfp7 sample to be 0.239 nmol per well (**Figure 3.3**). According to the amino acid analysis, the concentration of Dbfp7 sample used was 0.42 ug/ul.

In this experiment, 5 ul of Dbfp7 was used, therefore the total amount of the sample per well was:

$$0.42ug/ul * 5ul = 2.1ug/well$$

Considering Dbfp7 has a molecular concentration of approximately 8000 g/mol from the SDS-PAGE reading, the moles of Dbfp7 per well obtained was:

$$2.1ug * 10^-6/8000g/mol = 0.0000002625mol = 0.2625nmol/well$$

Dividing the moles of phosphate by the moles of Dbfp7 per well, we obtained:

$$0.2390nmol/0.2625nmol = 0.9105(91\%)phosphate/Dbfp7$$

Considering the solution containing Dbfp7 used was not ddH2O but 5% acetic acid, a control trial including 5% acetic acid as blank and different concentrations of bovine serum albumin proteins(BSA) as a negative control was conducted. The absorbance values obtained for either ddH2O or 5% acetic acid are both on the lower end of the standard curve, which are negligible (**Figure A.4**). Therefore, ddH2O and acetic acid are predicted to have similar performance serving as blank for Dbfp7 samples, rendering the results obtained from the original experiment authentic.

The results indicate that out of all the Dbfp7 molecules in the sample, 91% of them were phosphorylated, which can also be interpreted as phosphorylation is presented in Dbfp7 by approximately one phosphate per protein. In justification of this result, we investigated an online prediction tool Predictor of Naturally Disordered Regions(PONDRs), a feed forward neural networks that use sequence attributes taken over windows of 9 to 21 amino acids in making the prediction. The experimental results demonstrated correspondency to the prediction that one tyrosine residue is phosphorylated at the sequence of Dbfp7-V14 [45].

3.3.3 Non-Linear Relationship between Phosphorylation and Concentration of Dbfp7

Furthermore, we hypothesized that the percentage phosphate will change linearly with the change in protein concentrations. Another trial of the experiment that used different concentration of Dbfp7 and BSA was performed, with all standard curve and samples prepared in 5% acetic acid to establish consistency.

From the line of best fit ($y = 0.2622x + 0.1851$ $R^2 = 0.9993$) generated from the phosphate standard absorbance (**Figure 3.4; Figure A.5**), the phosphate level of the Dbfp7 sample is determined to be 0.23376 nmol and 0.39969 nmol per well for 2.5 ug and 5 ug Dbfp7 protein respectively. Similar calculations as previous trial have been performed, and the resulted percentage phosphate were 89% and 76% per Dbfp7 respectively. This result further demonstrates that phosphorylation is presented in Dbfp7 by approximately one phosphate per protein. On the other hand, the percentage phosphate does not have a linear relationship with the concentration of Dbfp7 observed in the results, which counteracts our original hypothesis. One of the possible reasons lies behind this result is that phosphoylation does not occur for every Dbfp7.

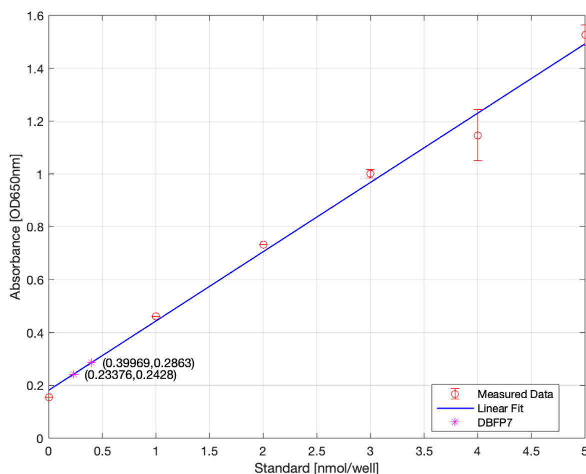


Figure 3.4: Trial 2 Phosphate absorbance of Dbfp7. Line of best fit generated from standard curve with absorbance(OD650nm) vs. phosphate concentration (nmol/well).

3.4 Limitation and Future Study

There are a few limitations of the protein extraction method. Despite the byssal protein of interest are collected in S1 and S2 extraction buffers, A few intracellular proteins also present in these extracts as the full phenol glands were homogenizing in the procedure. Another limitations comes from the procedure of SDS-PAGE. This is due to how proteins travel through the gel is also affected by the general structure of the protein. Specifically, charge on protein that is not paired with an SDS molecule and protein partners may affect how far the protein flies on the gel. Therefore, some protein bands shown on the gel can not be identified as which specific proteins according to molecular weight.

Potential improvements can be achieved through further investigation of the experiments performed in this research. Regarding Pro-Q Phosphoprotein Gel Stain, it is discovered a total protein SYRO stain along with this stain can be utilized to account for the weak phosphate standard bands and improve staining efficiency in future studies. In addition, quantitative analysis though Phosphate Assay can be further optimized using a larger concentration of Dbfp7 proteins in order to provide a more authentic result.

Further investigation in the detection of the glycosylation can be performed using Pro-Q glycoprotein stain for characterization of glycosylation [46]. This method allows the direct, in-gel detection of glycan groups similar to phosphate group detection done in this research. Furthermore, Periodic acid-Schiff staining can also be utilized as the next step for further sophisticated analysis of glycosylation in quagga mussel proteins[39].

Chapter 4

Conclusion

Overall, this research investigates the underwater mechanism of invasive freshwater mussel including quagga mussel *Dreissena bugensis* and zebra mussel *Dreissena polymorpha* discovered in the Great Lakes. An optimization based on buffer selection of zebra mussel byssal protein extraction is described in the first part of this thesis. Following determination upon the buffer that exhibits the highest extraction capacity, an amount of 1 ug of protein samples need to be collected and sent for further sophisticated analysis including amino acid analysis, liquid chromatography and mass spectrometry for a better demonstration of different adhesive protein identification and enrichment in each section of the byssus.

The second part of this thesis demonstrated a staining-based approach on the characterization of posttranslational modifications in quagga mussel protein. The results have suggested that there are several quagga mussel proteins that are post-translationally modified. Specifically, there is DOPA (as identified by Rzepecki and Waite [6]), showing evident phosphorylation by approximately one per protein, and potential glycosylated proteins. Althoug the non-linear relationship between phosphate concentration and Dbfp7 concentration suggested not every Dbfp7 had been phosphorylation-modified. These promising results can to be further supported by colourimetric/fluorescence spectrometry with DOPA/phosphate standards. Glycosylation can be specifically detected via glycosylation-specific stains and lectin blotting. The feasibility and performance of the methods can also be further investigated and improved in future studies.

Through a coherent understanding of the chemical nature of this attachment and adhesive proteins, this thesis presents considerable significance in the field of biomaterials, especially bioadhesives. Underwater adhesion mechanism utilized in freshwater mussel provides a well-established role model in the advancement of novel non-DOPA-dependent water resistant bioadhesives such as self-healing materials for wound closure and advanced coatings in aqueous environment.

Appendix A

Additional Experimental Results

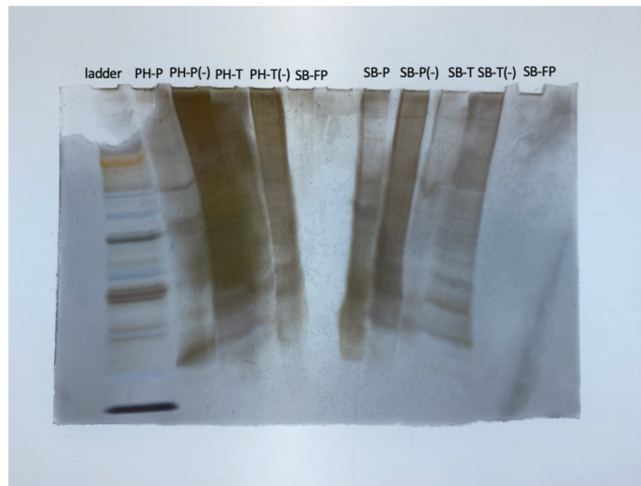


Figure A.1: Trial 2 Silver-stained gel image with zebra mussel byssal protein

Sample	Micromolar Concentration(uM)
Borate(SB8)	8.12
Borate(SB12)	9.57
Phosphate(PH12)	10.62
AcH12	0.88

Table A.1: Table of quagga mussel protein concentration

Sample	Micromolar Concentration(uM)
Blank	-0.11
(SB)T	1.21
(SB)P	17.71
(SB)FP	2.37
(PH)T	2.70
(PH)P	37.73
(PH)FP	0.43
(AcH)P	4.64
(AcH)T	0.89
(AcH)FP	2.37

Table A.2: Table of trial 1 zebra mussel byssal protein concentration

Sample	Micromolar Concentration(uM)
Blank	0.08
(SB)T	1.49
(SB)P	21.71
(SB)FP	3.19
(PH)T	1.49
(PH)P	27.40
(PH)FP	0.68

Table A.3: Table of trial 2 zebra mussel byssal protein concentration

	STD 1	STD 2	Sample	x
0	0.1261	0.1353	0.2222	0.239
1	0.4369	0.4537	0.2294	
2	0.6879	0.7728		
3	1.0552	1.0775		
4	1.2170	1.3432		
5	1.4574	1.6351		

Table A.4: Table of data showing absorbance of standard curve and samples obtained from the plate reader (OD650nm) * value x is the phosphate concentration (nmol/well) generated from the line-of-best-fit predicted in Figure 3.3.

	STD 1	STD 2	BSA2(AcH)	BSA5(AcH)	BSA10(AcH)	Dbfp7(2.5)	Dbfp7(5)
0	0.1557	0.1558	0.157	0.1649	0.1843	0.2384	0.2846
1	0.461	0.4611	0.1522	0.1605	0.1753	0.2472	0.288
2	0.7324	0.7314					
3	0.9886	1.0121					
4	1.2154	1.0778					
5	1.4982	1.5535					

Table A.5: Table of data showing absorbance of standard curve and samples obtained from the plate reader (OD650nm) * value x is the phosphate concentration (nmol/well) generated from the line-of-best-fit predicted in Figure 3.4.

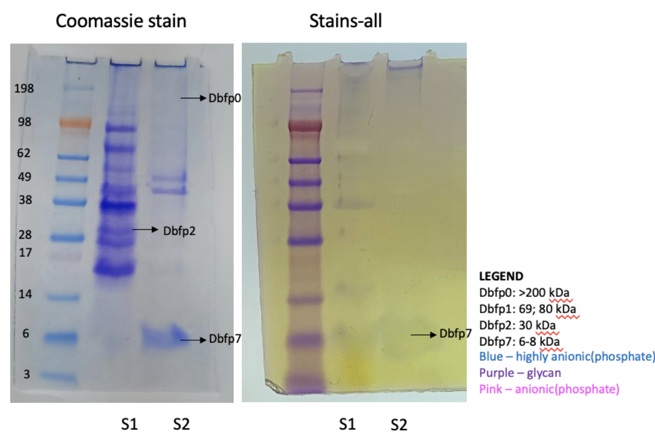


Figure A.2: Trial 1 Stains-all-stained gel image with quagga mussel protein S1 and S2 (Left: Coomassie stain; Left: Stains-all).

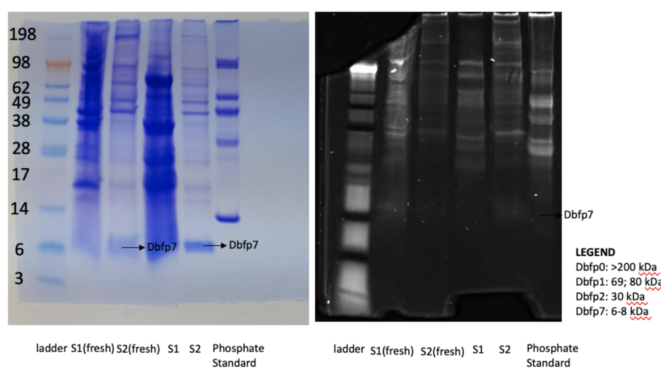
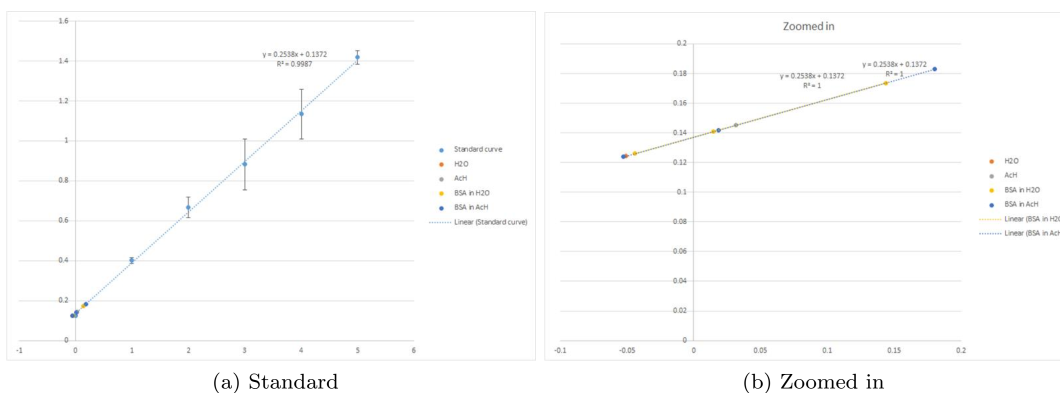


Figure A.3: Pro-Q Phosphoprotein gel image with quagga mussel protein S1 and S2 (Left: Coomassie stain; Right: Pro-Q Phosphoprotein stain



(a) Standard (b) Zoomed in

Figure A.4: Control trial. Line of best fit generated from standard curve with absorbance(OD650nm) vs. phosphate concentration (nmol/well).

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