AN INVESTIGATION INTO THE USE OF MUSSEL ADHESIVE PROTEINS AS TEMPORARY CORROSION INHIBITORS FOR HY80 STEEL

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ABSTRACT

AN INVESTIGATION INTO THE USE OF MUSSEL ADHESIVE PROTEINS AS TEMPORARY CORROSION INHIBITORS FOR HY80 STEEL

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Several proteins found in the adhesive system of the common blue mussel *Mytilus edulis* have chemical properties which might enable them to inhibit the flash rusting of steels. In this work, *Mytilus edulis* foot proteins (MeFPs) 1, 3, and 5 were purified and applied to HY80 steel in a number of buffer systems containing various amounts of borate, acetate, and phosphate at pH 5.5-7.0. Treated HY80 samples were then monitored in an exposure chamber at 40°C and 100% relative humidity for 7 days. The MeFP treatments were also evaluated electrochemically via electrochemical impedance spectroscopy (EIS). The effect of enzymatic crosslinking of the applied proteins using mushroom tyrosinase was also investigated. HY80 samples treated with MeFP-1 failed to inhibit corrosion when the protein was dissolved in deionized water, and the effect of MeFP-1 on the corrosion on HY80 in buffers containing acetate was not significantly different from the control samples. However, in 0.05M phosphate buffer solution at pH 5.5, crosslinked MeFP-3 and MeFP-5 were capable of significantly increasing the time to corrosion and
significantly reducing the mass loss of HY80 in the exposure chamber compared to relevant buffer-only controls. The performance of crosslinked MeFP-5 was similar to a commercial temporary corrosion inhibitor applied at the same mass concentration as the protein, suggesting that MeFP-5 and similar proteins or polymers may be capable of inhibiting corrosion under certain conditions.
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LIST OF ABBREVIATIONS AND NOTATIONS

BSA  Bovine Serum Albumin
CPP  Cyclic potentiodynamic polarization
DI   De-Ionized
DHBA L-3,4 dihydroxybenzoic acid
$E_{\text{corr}}$  Corrosion potential
$\Delta E_{\text{hys}}$  Hysteresis change in potential
EIS  Electrochemical Impedance Spectroscopy
$E_{\text{pit}}$  Pitting potential
$E_{\text{repass}}$  Repassivation potential
HPLC High-performance liquid chromatography
$I_{\text{corr}}$  Total Corrosion current
L-dopa L-3, 4 dihydroxyphenylalanine
MeFP  *Mytilus edulis* foot protein
PAGE  Polyacrylamide gel electrophoresis
pI   Isoelectric Point
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<th>Symbol</th>
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<tr>
<td>$\text{pH}_{\text{pzc}}$</td>
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<td>$R_p$</td>
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CHAPTER 1

INTRODUCTION

1.1 Background

Modern ocean going ships are protected from the corrosive effects of seawater by complex, multilayered coating systems that can last as long as twelve years before stripping and reapplication of the coating is necessary\(^1\). For a coating system to have that kind of longevity, it must uniformly and strongly adhere to the underlying metal hull of the ship. Unfortunately, the presence of contaminants such as particulates, soluble salts, and corrosion products can interfere with the adhesion of coatings to metal surfaces\(^2\). This reduces the useful life of the coating system. Therefore, before a coating system can be applied or reapplied, the surface must first be prepared by removing these contaminants. The most common type of surface preparation to remove coatings on metal surfaces is ultra-high pressure (UHP) water jetting, which leaves behind a metal surface that is clean and rough, but also wet\(^3\). Steel surfaces treated with UHP water jetting are well suited to accept new coatings, but they are also susceptible to a type of corrosion referred to as flash rust.

Under conditions of high humidity, water vapor present in the atmosphere can adsorb onto a metal surface, forming a thin film of water\(^4\). The formation of this thin film is the
key event that initiates flash rusting\textsuperscript{3}. The relative humidity above which the film is capable of forming is called the critical relative humidity. The value of the critical relative humidity varies greatly depending on the conditions of both the metal surface and the atmosphere\textsuperscript{2}.

In particular, the presence of soluble salts such as sodium chloride on a metal surface encourages the adsorption of water vapor, which lowers the critical relative humidity\textsuperscript{2}. For this reason, marine atmospheres are uniquely aggressive corrosion environments. Flash rust can easily form on a ship’s hull during the period between surface preparation and the first application of primer, and this corrosion can greatly reduce coating performance, increasing maintenance costs\textsuperscript{5}. Therefore, there is a need for an environmentally friendly temporary corrosion inhibitor capable of protecting the metal hull from flash rust during this time, while also promoting the adhesion of subsequent coatings. It is speculated that a group of proteins harvested from the attachment system of the blue mussel Mytilus edulis can perform in this capacity. The objective of this work is to evaluate the anticorrosive performance of Mytilus edulis foot proteins on high-strength, low-alloy steel. If these proteins prove to be effective corrosion inhibitors, then it is possible that a synthetic corrosion inhibitor could be developed based on the proteins’ unique chemistry.

\textit{1.1.1 Mytilus edulis Foot Proteins (MeFPs)}

Mytilus edulis is an immobile invertebrate filter-feeder that resides in the region of the coast between high and low tide. These mussels spend their lives attached to a variety of surfaces underwater including rocks, plants, man-made objects, and the shells of other
mussels (see Figure 1.1). The fact that Mytilus edulis can enjoy this kind of flexibility in an underwater environment despite the force of the waves is impressive, as the high dielectric constant of water renders the electromagnetic forces responsible for intermolecular attraction as much as 80 times less effective than they would be in air\textsuperscript{6}.

Mytilus edulis achieves these feats of underwater attachment by means of a group of flexible threads, which extend from a stem attached to the base of the mussel’s foot organ and terminate in small adhesive disc-like structures known as plaques\textsuperscript{7}.

![Mytilus edulis specimen](image)

**Figure 1.1:** A Mytilus edulis specimen suspended from a glass plate, to which several of its byssal threads are attached. Photo courtesy of D.C. Hansen.

Collectively, this attachment system is referred to as the byssus. The byssal threads themselves are composed of a thread of collagen-like proteins anchored to a network of more specialized adhesive and structural proteins in the plaque\textsuperscript{8}. Six of these proteins have been identified thus far; they are referred to collectively as *Mytilus edulis* Foot
Proteins (MeFPs) and they are numbered 1-6 in order of their discovery. The structure and function of each of these proteins will be discussed in detail in section 1.2.1.

1.1.2 L-DOPA

The most distinctive characteristic of the MeFPs is the presence of the unique post-translationally modified amino acid L-3,4-dihydroxyphenylalanine (L-dopa). L-dopa (Figure 1.2) is a modified version of tyrosine with an additional hydroxyl group adjacent to the first, forming a catechol functional group.

![Figure 1.2: L-dopa with the catechol functional group indicated](image)

One unique property of the catechol functional group is its ability to bond very strongly with metal ions. Catechols can bind to metal ligands with both of their adjacent hydroxyl groups, forming bidentate complexes with remarkably high stability complexes, particularly in the case of Fe(III)\(^9\). The complexes formed can be mono-, bis-, or tris-, as shown in Figure 1.3. These metal-catechol complexes can form either in solution, or on a metal oxide surface\(^{10}\). It has been shown that in some cases the formation of stable organometallic complexes on a metal surface can inhibit corrosion by preventing the dissolution of the oxide layer to which it is adsorbed\(^{11}\). Previous investigations have
indicated that L-dopa-containing proteins are capable of inhibiting corrosion to various degrees on some metals$^{12-16}$.

**Figure 1.3:** The three coordination modes of Fe(III) and catechol and the pH at which they are predominant. Information on the complexes is taken from Sillen and Martell$^{17}$. Image courtesy of D.C. Hansen.

The other important property of the catechol functional group relevant to its proposed incorporation into a corrosion inhibitor is its ability to participate in crosslinking reactions. These crosslinking reactions can be intermolecular or intramolecular. As a result of chemical or enzymatic treatment, the catechol present on L-dopa can undergo oxidation to form a quinone-intermediate, which can in turn react further with catechol groups or nucleophiles such as lysine present elsewhere in the protein to form a crosslinked protein$^{18,19}$. Intermolecular crosslinking increases the molecular weight of a single protein chain. A coating consisting of crosslinked protein will be more dense and more resistant to displacement, similar in effect to the curing of an epoxy resin$^{2,20}$. As an
adsorbed protein layer crosslinks and becomes more compact, it should inhibit corrosion by reducing the transport of water and ions through the protein film.

By utilizing both the metal-binding and crosslinking properties of the catechol functional group, proteins containing catechols such as the MeFPs may make effective corrosion inhibitors. Ideally, catechols adsorbed to a metal surface would form stable surface complexes that would promote the stabilization of the metal oxide, while any catechol functional groups not involved in the adsorption process would be available for crosslinking, which would lead to a more cohesive, multi-layer coating. If a naturally-derived, water-soluble, catecholic protein such as MeFP-1 can be shown to effectively inhibit corrosion in this fashion, it would be a valuable inspiration for the development of organic corrosion inhibitors.

1.1.3 Scope of this Research

In this work, the effectiveness of various Mytilus edulis foot proteins at inhibiting the flash rusting of HY80, a high-strength, low-alloy steel, was investigated. MeFPs 1, 3 and 5 were purified and their effects on the corrosion of HY80 were explored in an exposure chamber, as well as electrochemically by means of electrochemical impedance spectroscopy (EIS).

1.2 Literature Review

As discussed earlier, the attachment system of the mussel Mytilus edulis is termed the mussel byssus, and it is composed of a group of thin proteinaceous filaments called byssal threads which extend from the foot organ of the mussel and terminate in an adhesive anchor section called the plaque. The structure of the byssal thread as well as
the location of the various *Mytilus edulis* foot proteins in the thread can be seen in Figure 1.4.

![Figure 1.4](image)

**Figure 1.4:** The byssal thread structure, with the locations of the foot proteins indicated.

As seen in Figure 1.4, the adhesive “primer layer” of the plaque that attaches the mussel to its underwater substrate connects to a structural “foam” region. This foam region is thought to give structural support to the byssal plaque, and connect the adhesive region to the collagenous fibers, which in turn connect back to the foot of the mussel. The byssal plaque and the byssal thread are both protected from the environment by a hard, waterproof outer layer.

The six MeFP proteins labeled in Figure 1.4 have distinct sizes, amino acid compositions, and functions within the mussel byssus. Therefore, there may well be differences in the
way they might function as corrosion inhibitors. The properties of MeFPs 1, 3 and 5 will be discussed in detail in the following sections.

1.2.1 MeFP-1

MeFP-1 is the largest of the proteins, with a molecular mass of approximately 110 kDa\textsuperscript{21}. It is rich in proline, tyrosine, serine, threonine, lysine and alanine, and it is highly repetitive as it contains 75-80 consecutive repeats of a decapeptide sequence\textsuperscript{22} (see Figure 1.5)\textsuperscript{16,22,23}. In this sequence, as well as the rest of the protein, some of the tyrosine residues have been post-translationally modified to L-dopa. On average, L-dopa residues constitute approximately 13\% of the protein, with variations in that amount depending on the degree of posttranslational modification, which can vary by as much as 50\%\textsuperscript{24}. In addition, many of the proline residues have been modified to \textit{trans}-4-hydroxyproline or \textit{trans}-2,3-cis-3,4-dihydroxyproline\textsuperscript{22,25}. From the abundance of L-dopa and the other hydroxylated groups (hydroxyproline, serine, threonine) as well as primary amines (lysine), MeFP-1 can adhere firmly to many different substrates including slate, silica glass, metals and PTFE\textsuperscript{8,26}.

Due to its abundance and ease of isolation compared to the other MeFPs, MeFP-1 is the most widely studied of all of the MeFPs discovered so far. Interest in the protein is fueled by the need to develop bio-compatible adhesives capable of functioning inside the human body\textsuperscript{27}, as well as by its potential use as a corrosion inhibitor. However despite the interest in its adhesive properties, its large size and crosslinking ability are more important to its role in the byssus. Crosslinked MeFP-1 forms a waterproof lacquer
around the outside of the thread and plaque that protects the inner structure of the byssal thread from the environment\(^8\).

*Figure 1.5:* The repeated decapeptide expressed in MeFP-1. Possible hydroxyl groups present to some degree as a result of post-translational modification are represented with \(\dagger\)\(^{16,22,23}\).

1.2.2 *MeFP-3*

MeFP-3 represents a family of similar smaller proteins (around 6 kDa) with a high L-dopa content of 21 mol\(^\%\)\(^28\). Other prominent amino acids in its composition include glycine and arginine, of which large portions are modified to 4-hydroxy-L-arginine\(^29,30\). It stands to reason that the hydroxyarginine residue would be a good adhesive functional group, since the positively charged guanidinium group can interact with a negatively charged surface via coulombic forces, while the hydroxyl group grants the protein an additional hydrogen bonding site. MeFP-3 and MeFP-5 are the only two proteins to have
been confirmed to be present at the plaque-substrate interface\textsuperscript{31}; this observation along with its high L-dopa and hydroxyarginine composition reinforce the notion that MeFP-3 functions primarily as an adhesive. MeFP-3 has a less repetitive sequence than MeFP 1 and MeFP 2, and there are also several genetic variants of it. There was some speculation that \emph{Mytilus edulis} was able to somehow tailor the sequence of MeFP-3 to the substrate\textsuperscript{30}, but it is at present unclear what causes any particular MeFP-3 variant to be expressed\textsuperscript{8}.

1.2.3 MeFP-5

MeFP 5 is similar in structure and suspected function to MeFP-3; it is small at 9.5kDa, has the largest L-dopa content of the mussel foot proteins at 27%, and is found with MeFP-3 directly attaching the plaque to the substrate\textsuperscript{32}. The primary structure of MeFP-5 closely resembles that of MeFP-3 with a few important differences. First, lysine is more prevalent in MeFP-5 (20% vs 5%) and arginine is less common. Secondly, MeFP-5 is 10% serine, and most of this serine is phosphorylated in the form of ortho-phosphoserine. Phosphoserines have been shown to adhere to calcium and calcium-containing surfaces, leading some to speculate that the inclusion of phosphoserine in MeFP-5 assists mussels in attaching to rocks and the shells of other mussels\textsuperscript{32}.

1.2.4 Adsorption of \emph{Mytilus edulis} Foot Proteins to Metal Surfaces

The term ‘adsorption’ refers to the initial process by which a substance sticks to a surface, whereas ‘adhesion’ refers to how strongly the adsorbing substance is bonded to the substrate. The former process is described by adsorption isotherms, which describe the amount of substance present on the surface at equilibrium as a function of the concentration of the substance in the bulk fluid, whereas the latter is described the pull-
off strength of a coating\textsuperscript{2,33}. How well a polymer adheres or adsorbs to a substrate strongly depends on the type of chemical interactions occurring between the molecule and substrate\textsuperscript{34}. These interactions vary in strength, with the strongest being the covalent bond, but weaker, more reversible interactions such as hydrogen bonding and cumbic interactions are also important. Metal-ligand bonds, particularly those involving L-dopa, are of special interest since they are of intermediate strength, but reversible (i.e. not as strong as covalent bonds but stronger than hydrogen bonds and other non-bonding interactions)\textsuperscript{35}.

Most metals form one or more oxide layers very rapidly when exposed to oxygen. The exact composition of these metal oxide layers in aqueous solution is often not perfectly understood, since it depends to some degree on the nature of both the metal and the solution. However, in general the outermost layer of a metal surface in aqueous solution terminates in a thin layer of metal hydroxide\textsuperscript{34,36}. Catechols have been shown to form surface complexes with these metal hydroxide layers by displacing the surface hydroxyl groups\textsuperscript{10,37,38}. But while metal-ligand surface complexation is the strongest form of attachment available to MeFPs, it is not the only trick up the mussel’s sleeve when it comes to metal oxide adhesion. For one thing, most of the proteins (particularly MeFPs 1 and 5) have a considerable amount of polar residues in their consensus sequences, which would allow for adsorption in the form of hydrogen bonding and other dipole interactions. In addition, all of the MeFP proteins have a high isoelectric point (~9-10.5)\textsuperscript{39} due to the large amount of basic amino acid residues present in their primary structure. As a result, at all pH’s less than ~9, the proteins will have a net positive charge, which allows for the possibility of coulombic attraction to a negatively-charged substrate.
Because metals are amphoteric, the charge of a metal oxide surface is dictated by the pH of zero charge \( (\text{pH}_{\text{pzc}}) \) of the thin hydroxide layer. Similar to a protein’s isoelectric point, at pH’s lower than the \( \text{pH}_{\text{pzc}} \), the oxide will be positively charged, and at pHs higher than the \( \text{pH}_{\text{pzc}} \) the oxide will have an overall negative charge. Therefore, the adsorption of proteins to a metal surface can be expected to vary with pH, as the net charge of the protein and metal surface will be determined by the pI and the \( \text{pH}_{\text{pzc}} \), respectively.

Previous research\(^{12}\) into the adsorption of MeFP-1 onto 304L stainless steel at pH 7.5 compared the adsorption of the mussel protein to that of a low molecular weight catechol, L-3,4 dihydroxybenzoic acid (DHBA); a highly-charged polymer, poly-lysine; and a globular protein, Bovine Serum Albumin (BSA). In addition to adsorption data in the form of adsorption isotherms, data regarding the amount of metal ions in solution after the 2 hour incubation period in the adsorbent was also gathered.

It was found that MeFP-1 had significantly more adsorption sites than BSA, and the highest affinity constant of all the adsorbents measured (although not significantly higher than BSA). DHBA had a much larger number of adsorption sites by far, due to its small molecular weight, but the lowest affinity constant. It was also noted that DHBA greatly increased the amount of metal in solution after 2 hours, compared with a control sample. This indicates that the catechol functional group on DHBA likely formed complexes with the surface metal, but the dissolution of these complexes resulted in metal ions being released into solution. This phenomenon has been observed before\(^{36,40}\). MeFP-1, on the other hand was found to greatly reduce the amount of metal ions in solution compared to its buffer control. Although the fact that the DHBA and MeFP-1 were in different buffers complicates the analysis somewhat, the fact that MeFP-1 is capable of significant
adhesion while reducing the amount of metal ions in solution lends some credence to the notion that it might act as a corrosion inhibitor.

1.2.5 Corrosion Inhibition Properties of *Mytilus edulis* Foot Protein

A few investigations have already been made into the potential use of MeFPs as corrosion inhibitors. So far, published data only exists for MeFP-1, and then only for a handful of substrates; stainless steel, aluminum, and more recently, carbon steel.

Early work\(^\text{13}\) into the effectiveness of mussel protein as a corrosion inhibitor compared the effectiveness of MeFP-1 as a corrosion inhibitor with BSA, DHBA, poly-lysine, and untreated controls via cyclic polarization experiments. Two experiments were done; in the first case the samples were incubated for 1 hour in a solution containing the respective compounds in 5% acetic acid at a pH of ~2.5, in the second; the inhibitors were dissolved in either a HEPES or borate buffer with a pH of 7.5-8.0.

At pH 2.5, MeFP-1 exhibited the least total charge passed out of all the treatments during cyclic polarization, significantly less than the controls. However, the differences in charge passed between MeFP-1 and the other treatments were not statistically significant. The amount and depth of the pits on the MeFP-1 treated samples were significantly less than both the controls and the other treatments at pH 2.5. The features of the polarization curve that were examined included corrosion potential ($E_{\text{corr}}$), pitting potential ($E_{\text{pit}}$), repassivation potential ($E_{\text{repass}}$), and the difference between $E_{\text{pit}}$ and $E_{\text{repass}}$, termed $\Delta E_{\text{hys}}$. There were no significant differences observed between the treatments in these measurements, at pH 2.5, except in the case of MeFP-1. Cyclic polarization curves on MeFP-1-treated 304L steel did not show a clear stable passive region as seen in the
controls; consequently no $E_{\text{pit}}$ value was measured at pH 2.5. The lack of a passive region in the polarization curve despite an apparent reduction in overall corrosion was presumed to be a result of electron transfer processes involving the L-dopa-quinone redox couple.

Inhibition was less apparent at pH 7.5. Here MeFP-1 showed significantly less charge passed during cyclic polarization compared to a blank control, but not compared to a relevant buffer control or the other treatments investigated. Other measures such as pit depth, mass loss also did not show significant differences between MeFP-1 and the controls or other treatments.

Further investigations\textsuperscript{15} into the effect of MeFP-1 on aluminum using Electrochemical Impedance Spectroscopy (EIS) showed that while non-crosslinked MeFP-1 did show the same reduction in passive current density and pitting potential during anodic polarization in 0.1M NaCl that had been observed in earlier research, it did not show an increase in polarization resistance ($R_p$) compared to the control. In contrast, the crosslinked protein at a 1.0 mg/mL (9.8x10$^{-6}$M) concentration, maintained an $R_p$ 2-3 factors of ten greater than the control over an 11 day period. These findings clearly show that enzymatic crosslinking of MeFP-1 can enhance the anticorrosive properties of MeFP-1.

In the past three years, several papers\textsuperscript{41-45} have been published from a group investigating the effectiveness of MeFP-1 on carbon steel. Unlike stainless steel and aluminum, carbon steel does not passivate under normal circumstances, and therefore corrodes uniformly and is not as susceptible to pitting. In this respect, carbon steel is perhaps more similar to HY80 than stainless steel or aluminum.
The earliest of these papers used EIS experiments to evaluate MeFP-1 as a free corrosion inhibitor, i.e. carbon steel samples were exposed to a corrosive solution that also contained 1mg/mL MeFP-1, rather than incubating the samples in a protein solution as was done in the other experiments discussed. The solutions used were 0.2M phosphoric acid adjusted to pH 4.6 with NaOH that was also 0.1M in NaCl. A small increase (less than one order of magnitude) in $R_p$ was reported after 7 days of immersion. While the increase in $R_p$ may have been statistically significant, it is likely too small to be of practical significance. In another paper, EIS was used to test carbon steel coupons incubated in a MeFP-1 solution (1mg/mL, 1% citric acid at pH 9.0). Chemical crosslinking was also tested in this study by treating the pre-formed film with sodium periodate (NaIO₄). Results in this study were similar to the first; both treatments showed a larger $R_p$ than the control after 30 minutes, with the crosslinked protein preforming slightly better, but the effect was small, and it did not persist with time.

Two more papers used repeated immersions in MeFP-1 followed by ceria nanoparticles, in an effort to facilitate multi-layer adsorption to a metal substrate. Despite the formation of a well-characterized film, no significant corrosion inhibition was observed initially. After seven days of immersion, however, the polarization resistance had increased by a factor of around 50 from the initial value recorded after one hour. Subsequent anodic polarization experiments showed that the corrosion current after 7 days of immersion was 98% less than the bare steel control; these values are in good agreement with the increase in $R_p$. The increase in corrosion resistance over time was attributed to metal-induced crosslinking in the MeFP-1/ceria composite film, resulting in
a denser, more thoroughly crosslinked composite film that formed a more effective coating.

1.2.6 Corrosion Inhibition with Catecholic Siderophores

Some studies have investigated the use of catechol-containing siderophores as corrosion inhibitors. Siderophores are small biomolecules generated by microorganisms for the purposes of binding soluble iron for other cellular purposes. In one paper\textsuperscript{46}, electrochemical experiments were done on pure iron in 1M HCL containing various concentrations of a siderophore. Parabactin, a siderophore containing two catechol groups and a phenol group, was the most effective of the 4 siderophores tested; a 1.4x10\textsuperscript{-4}M parabactin solution reduced the corrosion current (I\textsubscript{corr}) by 90% and an order-of-magnitude increase in R\textsubscript{p} compared to blank controls. The greater effectiveness of parabactin was attributed to its catechol functional groups and larger size compared with the other siderophores tested.

In another study\textsuperscript{16}, the effects of parabactin as well as pre-adsorbed MeFP-1 layers were investigated on aluminum in 0.1M NaCl solution. Both the mussel protein film and the saturated parabactin solution were capable of shifting the pitting potential of aluminum by roughly +80mV. In addition to the shift in the pitting potential, it is notable that mussel protein also decreased the passive current density by almost a factor of ten. This finding in conjunction with FTIR work\textsuperscript{47} showing that parabactin adsorbs to aluminum via the formation of organometallic complexes with the aluminum oxide surface reinforce the idea that adsorbed MeFP-1 is capable of stabilizing the oxide layer.
1.2.7 Effect of the Catechol Functional Group on Metal Oxides

Research relating to the effect of catechol functional groups on various metal oxides may also be relevant to this investigation. It has been hypothesized that catechols are capable of stabilizing a metal oxide layer by the formation of a surface complex\textsuperscript{13}. However, not all instances of metal-ligand surface complexation result in increased corrosion resistance. Chelation of a metal oxide surface by an organic ligand can result in either enhanced or reduced dissolution of metal oxides\textsuperscript{48,49}. The effect of metal ligand bonds on metal dissolution is thought to depend on the type of surface complex formed. In particular, binuclear surface complexes (complexes involving more than one metal atom) have been shown to inhibit dissolution\textsuperscript{49}. The removal of two metal atoms from a lattice is thought to be much more energetically unfavorable than the removal of one metal atom\textsuperscript{11}. MeFP-1 has been shown to form a binuclear complex with Fe\textsuperscript{3+} in solution at pH 7.0, and other binuclear catechol-iron complexes have been observed in nature\textsuperscript{50,51}. However, the type of bond that MeFP forms at an iron oxide surface is not known. Some studies of low molecular weight catechols on various metal oxide surfaces have shown that catechol enhances oxide dissolution\textsuperscript{52–54}, but at least one study of catechol adsorption on goethite found no detectable metal dissolution and a mix of mono and bidentate complexation\textsuperscript{55}. Studies investigating the effect catechol-containing polymers on iron oxide nanoparticles have shown varying levels of nanoparticle stability with different polymer formulations\textsuperscript{51}. Because the effect of catechol on metal oxides varies greatly with pH, oxide composition, and other factors\textsuperscript{51,53,55}, no easy direct comparison between results with low molecular weight catechols on minerals of uniform composition and experiments with MeFPs on oxide-covered metal surfaces can be made.
1.3 Hypotheses

The ability of the mussel proteins to inhibit corrosion will be evaluated by testing the following hypotheses:

**Hypothesis 1:** *Mytilus edulis* foot proteins 1, 2 and 5 inhibit the flash rusting of HY80 steel in the exposure chamber.

**Hypothesis 2:** Crosslinking will increase the amount of corrosion resistance that the MeFPs confer to HY80 steel by increasing the thickness and water-resistance of the MeFP films, resulting in better performance in the exposure chamber and higher polarization resistance as measured by Electrochemical Impedance Spectroscopy (EIS).

**Hypothesis 3:** Differences in the performance of the MeFPs will be directly related to the L-dopa content in the protein, as verified by amino acid analysis data.
CHAPTER 2
MATERIALS AND METHODS

2.1 Materials and Sample Preparation

HY80 plate with 0.25 inch thickness was obtained from Clifton Steel (Cleveland, OH) and cut into 0.6875 inch square coupons. These coupons were then ground in water to 400 grit, and then finished with ethanol at 600 grit. After grinding, samples were cleaned by sonication sequentially for 5 minutes each in acetone and then isopropanol, before being dried under a stream of argon and stored. Samples were stored in a heated incubator kept at 40°C to maintain a dry environment for 16-24 days until use. For the MeFP-1 experiments (section 2.4.5) samples were stored for only 24 hours prior to exposure. In the preliminary work, the storage time before treatment and exposure was not controlled. Samples used for exposure testing were masked with 3M #470 electroplating tape (3M, St. Paul, MN). A 0.5 cm² area of each sample was exposed.

2.2 Protein Extraction and Purification

The methods used in this research to isolate and purify MeFPs 1, 3, and 5 followed methods that have been described previously⁵⁶, but they will be discussed here in brief. *Mytilus edulis* feet were harvested from live specimens obtained from American Mussel Harvesters (North Kingston, RI), frozen at -80°C, and stored for later extraction. The
extraction itself consists of initial extraction of the proteins by blending mussel feet in a 0.7% perchloric acid solution, followed by the precipitation of the proteins with the addition of cold acetone, then resolubilization of the protein in 5% acetic acid. After extraction, the protein was then concentrated using ultrafiltration under inert gas in preparation for purification using size-exclusion chromatography.

A schematic of the purification scheme used for a majority of the protein used in this work is shown in Figure 2.1.

**Figure 2.1:** A schematic of the purification scheme used to isolate MeFP-1, 3 and 5

Size exclusion chromatography was preformed primarily with Sephadex G-200 and Sephracyl S-400 gel columns (GE Healthcare Bio-Sciences Corporation, Piscataway,
NJ). The G-200 was used for the initial separation of MeFP-1 from the other MeFPs, while the S-400 was used on mixed MeFP samples to separate the remaining MeFP-1 from mixtures of MeFP 2, 3 and 5. It was found that mixes of MeFPs 2, 3 and 5 can be easily separated by HPLC provided MeFP-1 was not present. Between purification steps, the protein was lyophilized and stored at -60°C. After each step in the process, purification was evaluated by acid-urea polyacrylamide gel electrophoresis (PAGE). The methods related to the electrophoresis gels used to visualize the composition of protein mixes has been described elsewhere. Data pertaining to the purity and amino acid composition of the protein used in these experiments is presented in Appendix A.

2.3 Amino Acid Analysis

The AAA-Direct Amino Acid Analysis System (Dionex, Sunnyvale CA) was used to determine the concentration of protein present in the samples used in the experiments, as well as the relative amino acid composition of those protein samples. Samples of protein were taken after the final purification step and diluted such that the concentration of protein was 1mg/mL. Ten microliter aliquots of each sample were hydrolyzed under vacuum with 6.0 M HCl containing 1.0 percent (by volume) phenol for 12-18 hours at 110°C.

2.4 Protein Treatments and Exposure Chamber Conditions

For the exposure chamber experiments, after the initial incubation period, treated HY80 samples were kept in a 100% humidity environment maintained at 40°C in a Q-FOG CCT-1100 exposure chamber (Q-Lab Corporation, Westlake OH) All samples were periodically removed and photographed so that the extent of corrosion could be
determined, then returned to the exposure chamber. After seven days of exposure the samples were removed from the chamber. Seven days was chosen as the exposure period because preliminary experiments showed that all samples treated with the commercial inhibitor began to corrode after seven days in the exposure chamber.

The time period before exposure when the various treatments were applied is referred to as the ‘incubation period’ of the treatment. Sections 2.4.1 - 2.4.8 describe the treatment methods used in the various experiments discussed.

2.4.1 L-dopa as a Free Amino Acid

In the cyclic polarization experiment, three HY80 coupons with a 60-grit finish were cleaned as described earlier. The samples were incubated in a 2.75mM L-dopa solution for 1 hour. After a three hour period during which the open circuit potential reached steady state, cyclic potentiodynamic polarization (CPP) scans were run. The cyclic polarization scans had a vertex voltage of 1.0V vs. open circuit, a scan rate of 10mV/s and a step size of 10mV. Tafel analysis and curve fitting was also done so that the total corrosion current ($I_{corr}$) could be measured. Tafel fits were made using the PowerSuite software package (Princeton Applied Research, Oak Ridge, TN), which performs linear curve-fitting to determine the cathodic and anodic Tafel slopes, allowing $I_{corr}$ to be determined. Natural seawater from the Naval Research Lab (Key West, FL) was used as an electrolyte for these experiments.

2.4.2 MeFP in Deionized Water

Three small exposure experiments are described here. In all cases, the incubations were done with protein dissolved in deionized (DI) water with no buffering agent. The pH of
the incubation solution was 4.0-5.0. In the first experiments (Figure 3.4), 0.5mL of a
3mg/mL solution of MeFP-1 was applied to an HY80 sample with a 60 grit surface
finish. The incubation solution was allowed to dry out overnight before being placed in
the exposure chamber. In the second experiment (Figure 3.5), an epoxy-mounted HY80
sample with a 600 grit finish was used. The sample was treated with a 1.5mg/mL solution
of mixed MeFP. The protein solution was pipetted away when a purple coloration was
first noticed, which in this case was after 35 minutes of incubation time. The sample was
then gently rinsed with deionized water and placed in the exposure chamber. In the third
experiment, (Figure 3.6), an epoxy-mounted HY80 sample with a 600 grit finish was
treated with a 1.5mg/mL MeFP-1 solution. After 1 hour of incubation, the sample was
additionally treated with 10µL of a mushroom tyrosinase solution (E.C. #1.14.18.1,
Sigma-Aldrich). After an additional hour of incubation, the excess incubation solution
was pipetted away and the sample was placed in the exposure chamber.

The second and third experiments, as well as the preliminary phosphate exposure
experiments discussed in section 3.1.4 used epoxy-mounted HY80 samples rather than
samples masked with tape. This was done out of concern regarding tape delamination
affecting the results. However, epoxy mounted samples proved to be vulnerable to
crevise corrosion at the metal-epoxy interface, and their use was discontinued. Tape
delamination, when it did occur, occurred with samples that had already greatly corroded,
and the areas of delamination were usually small in comparison to the exposed area, so
these samples were still considered in the data analysis. Corrosion did not initiate under
the tape, and although it often initiated at the edge of the taped area, this is also the area
where the samples were most often wet. Samples treated with borate-acetate buffer or
commercial inhibitor often went several weeks in the chamber, with none of the non-
corroding samples experiencing corrosion, and when corrosion did occur, it did not
necessarily begin at an edge. This reinforces the notion that the delamination of the tape,
when it occurred, was an effect of sample corrosion, rather than a cause.

2.4.3 MeFP-1 in Borate-Acetate Buffer

In the borate-acetate buffer experiments, a buffer was prepared from 5% acetic acid that
was also 0.05M in borate. This buffer was adjusted to pH 7.0 with 10M NaOH, and then
mixed with an equal volume of deionized water containing MeFP-1 at a 2mg/mL
concentration. The final buffer concentration was 0.437M in acetate and 0.025M in
borate. The final MeFP-1 concentration was 1 mg/mL based on the dry weight of the
protein. The protein layer was incubated for 24 hours, allowing the protein layer to dry
out. Some of the protein samples were additionally treated with 10 microliters of a
10mg/mL mushroom tyrosinase solution in the same buffer. These protein samples were
compared with an equal number of control samples treated with borate-acetate buffer.
0.3mg of MeFP-1 total was applied to each sample at an initial concentration of
2.6mg/mL. The HY80 samples used were polished to a 1200 grit finish. The length of the
experiment was 30 days.

2.4.4 Preliminary Phosphate Buffer Experiments

For these experiments, 1.13mg of pure MeFP-1 was dissolved in DI water and mixed
with an equal volume of phosphate buffer such that the solution applied to the samples
was 1.13mg/mL MeFP-1 and 0.05M in phosphate. The pH of the solution was 7.0. For
this and the subsequent phosphate buffer experiments, protein films were not completely
dried out during the incubation process. After 1 hour of incubation, the excess solution was pipetted away and an unbuffered 1mL solution of mushroom tyrosinase (E.C. #1.14.18.1, Sigma-Aldrich) containing 1,100,000 units of catechol oxidase activity per milliliter was added to the surface of the sample. After an additional hour of incubation, this solution was also pipetted away and the samples were placed in the exposure chamber. The buffer controls were similarly incubated, except the solutions did not contain protein or enzyme. In this experiment, four enzyme-treated protein samples were compared with ten controls. Some of the samples in each treatment group were prepared at different times; half of the samples in the control group were prepared 14 days before the experiment, while the other half was prepared 2 days before the experiment. Two of the protein-treated samples were prepared 2 days before the experiment, while the other two protein-treated samples were prepared 14 and 28 days before the experiment. The samples prepared 2 days prior to experimentation are referred to as the ‘fresh oxide’ samples, while the other samples are referred to as ‘aged oxide’ samples.

2.4.5 MeFP-1 in Phosphate-Acetate Buffer

In the MeFP-1 experiments, 0.5mg of protein per sample was first dissolved in a small volume of 0.1M acetic acid in an attempt to increase the solubility of the protein. After the addition of 0.05M phosphate buffer, the pH of the solution was then adjusted with 0.5M NaOH. The final concentration of the buffer was 0.01M acetate, 0.045 M phosphate at pH 6.0. The final concentration of the protein solution applied to the samples was 1mg/mL. Enzyme treated samples were treated with the same enzyme solution described in section 2.4.6, except that no buffering agent was added to the enzyme solution.
2.4.6 MeFP-3 and MeFP-5 in Phosphate Buffer

For the exposure chamber experiments, the purified, freeze-dried MeFP proteins were dissolved first in cold DI water to a concentration of 2mg/mL (with the protein concentration being based on the dry weight of the lyophilized protein). The protein was stirred and kept in the refrigerator for 10-15 minutes to give the protein time to fully dissolve, and then an equal volume of 0.1M phosphate buffer at pH 5.5 was slowly added to the protein solution. Therefore, the solution added to the metal coupons was a 1mg/mL protein solution dissolved in 0.05M phosphate buffer. The added protein solution did not change the pH of the buffer solution more than 0.05 pH units.

Each of the masked HY80 samples had an exposed area of roughly 0.5cm², which was treated with 0.5mL of the protein solution for one hour, after which the excess protein was carefully removed via pipetting. After this incubation period, the samples were photographed and placed in the exposure chamber to begin the experiment.

Some protein samples were further treated with a crosslinking enzyme, mushroom tyrosinase (E.C. #1.14.18.1, Sigma-Aldrich). After the excess protein was removed via pipetting, a 25µL aliquot of tyrosinase enzyme with a total catechol oxidase activity of 275,000 units was dissolved in DI water was mixed with 25 µL of 0.1M phosphate buffer at pH 5.5. The resulting solution was applied to the sample and allowed to incubate for an additional hour before being pipetted away. Following the enzyme incubation period, the samples were photographed and placed in the exposure chamber.

In the MeFP-3 experiments, sodium periodate was also used to crosslink MeFP-3. The same batch of MeFP-3 was used for these experiments as the other MeFP-3 experiments.
It was desired to use a concentration of periodate equimolar to the amount of L-dopa in the protein that needed to be crosslinked. This concentration was determined to be 0.096mM via the Arnow assay for catechols. Both the periodate-treated controls and the periodate-treated MeFP-3 samples were treated with 0.5mL of this concentration of periodate.

2.4.7 EIS Experiments

The protein incubations for the electrochemical experiments followed the same protocol as the exposure chamber samples; in the case of MeFP-1, the method was the same as described in section 2.4.5, while for MeFPs 3 and 5, the method was identical to section 2.4.6. However, in the EIS experiments, instead of being masked with tape the HY80 samples were treated with protein after having already been clamped into the working electrode position of the electrochemical cell (exposed area was 0.5 cm²). After incubation, the cells were slowly filled with 0.1M NaCl and allowed three hours for the open circuit potential to stabilize before the first measurements were taken.

2.4.8 Commercial Inhibitor

The commercial flash rust inhibitor used for comparison in these experiments was Halox® 900 (ICL Performance Products, St. Louis MO), a corrosion inhibitor recommended by the manufacturer for use as a temporary corrosion inhibitor for ferrous and nonferrous metal parts during storage. It is supplied as concentrated liquid that can be used in various dilutions. For these experiments, it was applied at an equal mass concentration as the protein with which it was to be compared with (1mg/mL, based on the solids content of the inhibitor listed by the manufacturer).
2.5 Methods of Evaluation for Exposure Chamber Experiments

Image analysis was used to determine the amount of the exposed samples’ surface covered with corrosion product at certain time intervals. This was done using a color threshold technique using the ImageJ software package (National Institute of Mental Health, Bethesda, Maryland, USA). ImageJ is capable of identifying and measuring pixels based on color in the hue-saturation-value color model. Parameters of the color model were chosen such that the software would reliably identify all of the corroded areas of the sample. This area (in pixels) could be compared with a user-defined area representing the entire exposed area of the sample, giving a quantitative value for the portion of the surface covered with corrosion. Similar methods have been used before\(^3\). The ratio of rust-colored (red or yellow) pixels to the total number of pixels in the exposed area is defined as the “percent inhibition”.

\[
\left(1 - \frac{(red \ or \ yellow \ pixels)}{(total \ pixels \ in \ exposed \ area)}\right) \times 100\% = percent \ inhibition
\]

In addition to the image analysis, the effectiveness of the protein treatments in the exposure chamber was also judged by the time until the first visible corrosion was observed (time to corrosion), and the mass loss data, measured after the conclusion of the experiment. Corrosion products were physically removed from the samples via abrasion by a mild scouring pad and cleaned by sonication in isopropanol. After the mass was recorded, the cleaning procedure was repeated until subsequent cleanings did not result in a change in mass, in a manner consistent with ASTM G1\(^5\). Statistical analysis for this data consisted of using a one-way ANOVA to determine if there was a significant difference between the treatments tested. If a significant difference was detected, the
Tukey-Kramer method was used as a post-hoc test to determine which differences were significant. In both the ANOVA and Tukey-Kramer statistical analyses, differences in treatment means were considered significant if $p<0.05$.

2.6 **Electrochemical Impedance Spectroscopy (EIS) Experiments**

Electrochemical Impedance Spectroscopy is a technique whereby a small-amplitude AC signal is applied to a metal-electrolyte system and the system’s impedance response is analyzed. It is widely used to study the corrosion behavior of coated metals in aqueous environments, in part because it is a nondestructive technique\textsuperscript{34}.

![Experiment setup](image)

**Figure 2.2:** The type of electrochemical cell and reference electrode used in the EIS and polarization experiments

After the sample incubation period was complete, samples used for the electrochemical experiments were used as the working electrode in an electrochemical cell setup (see Figure 2.2). The same model of cell was used for the polarization measurements on
L-dopa. A Solartron SI 1287 potentiostat in conjunction with a Solartron SI 1260 frequency response analyzer was used to make measurements on multiple cells sequentially by means of a Scribner Associates 314 Multiplexer (Southern Pines, NC). In this experiment, six electrochemical cells containing the samples prepared as described in section 2.1 were monitored at open circuit for 5 days, during which time EIS experiments were conducted every 24 hours. The polarization resistance (R_p) was determined by subtracting the low-frequency limit of the total impedance from the high-frequency limit, which was taken as the solution resistance (R_s). A three hour equilibration period was sufficient time for the open circuit voltage to stabilize (±10mV/hr), after which EIS experiments were run periodically over the course of the five-day experiment. The frequency range scanned was 100 kHz to 0.01Hz and the amplitude of the excitation signal was 10mV rms.

2.7 X-Ray Photoelectron Spectroscopy (XPS)

The XPS technique consists of bombarding a sample with X-rays of a known energy. When the X-rays are absorbed by atoms in a material, electrons are released. The kinetic energy of these electrons is a function of the energy of the incoming X-rays and the type and oxidation state of the atom that released them. XPS gives information about the identity, relative quantity, and chemical bonding state of atoms present on a surface in the nanometer range\textsuperscript{60}. In this work, XPS was used to analyze the differences in the air-formed oxide of HY80 that developed between grinding and cleaning of the samples and their subsequent use in experiments. Two samples were examined, with one being stored in lab air for two days, and another being stored for 17 days.
A SSX-100 XPS instrument (Surface Science Laboratories, Mountain View, CA), capable of producing a vacuum of $10^{-9}$ torr was used for these experiments. A nominal x-ray spot size of 600 µm was used for the analysis. Two spots were analyzed on each sample. A survey scan from 0 to 1000 eV and high resolution scans of carbon, oxygen, iron, manganese, nitrogen, and chromium binding energies were taken.
CHAPTER 3

RESULTS

3.1 Preliminary Work

The earliest experiments investigated the inhibition effectiveness of the free amino acid L-dopa. After sufficient quantities of the mussel proteins had been purified, exposure chamber experiments were conducted with protein in a variety of buffer systems to determine what conditions of buffer chemistry and pH would allow the protein to effectively inhibit corrosion. The results of exposure chamber experiments with protein dissolved in deionized water, borate-acetate buffer and early experiments with phosphate buffer are included in this section.

3.1.1 L-dopa as a Free Amino Acid

The first experiments that were conducted tested the anticorrosive effectiveness of L-dopa electrochemically as well as in the exposure chamber. The electrochemical experiments indicated that free L-dopa did not have any lasting inhibitory effect on HY80 after 3-hour immersion in seawater (Figure 3.1). These findings are in agreement with previous work done with low molecular weight catechols at neutral pH by Hansen et. al.\textsuperscript{12,13}, in which DHBA did not significantly reduce the corrosion of S30403 stainless steel, and increased the dissolution of iron and chromium from 304L stainless steel.
**Figure 3.1:** Mass loss and $I_{\text{corr}}$ data from cyclic polarization experiments on HY80 samples treated with L-dopa. Error bars are one standard deviation. ANOVA analysis revealed no significant difference between the treatments.

Preliminary exposure data in which HY80 samples were treated with a 10mM L-dopa solution before being placed in the exposure chamber (Figure 3.2) showed that the L-dopa treated samples did not inhibit corrosion, which agrees with the electrochemical findings.
Figure 3.2: Representative HY80 samples from an exposure chamber experiment after a) one hour and b) 9 hours of exposure in a 40°C, 100% relative humidity environment. Samples were ground to a 60-grit finish and were incubated with in their respective solutions for 1 hour before being placed in the exposure chamber.

Figure 3.3: 10mM L-dopa solution applied to a 600 grit HY80 surface after 20 minutes of incubation.

HY80 samples treated with L-Dopa at pH 7.0 very quickly turned a purple color, likely due to the formation of catechol-iron complexes (see Figure 3.3)\textsuperscript{61}. 

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3.1.2 MeFP in Deionized Water

A few exposure chamber experiments with a small sample size were carried out in which MeFP-1 was dissolved in de-ionized water and applied directly to HY80 steel, without any pH buffering agent. The pH of the incubation solution in these experiments was estimated to have been between 4.0-5.0 based on pH meter readings and readings from pH paper. The degree of uncertainty regarding the pH is due to the unreliability of most pH meters when measuring the pH of solutions with very low ionic strength\textsuperscript{62}. Some acidification has been observed during the course of this work in other buffer systems as a result of the addition of lyophilized protein. This could be a result of the so-called “pH memory effect”, in which lyophilized proteins retain the protonation state of the buffer they were lyophilized in\textsuperscript{63}. Because the buffer used for lyophilization was most commonly 5% v/v acetic acid, this would explain the observed acidification.

Three incubation schemes were attempted with MeFP-1 in deionized water: in the first experiment (Figure 3.4), MeFP-1 films were allowed to incubate for 24 hours, allowing them time to fully dry out; in the second (Figure 3.5), the protein was allowed to incubate for 35 minutes before being gently rinsed with deionized water; and in the third (Figure 3.6), the protein was incubated for an hour before being additionally treated with 10µL of a mushroom tyrosinase solution with 29,000 unit/µL of catechol oxidase activity for additional hour before the solution was wicked away and the samples were exposed in the chamber.
Figure 3.4: Incubation of pure MeFP-1 dissolved in deionized water on HY80 steel. The amount of protein applied to the sample was 3mg/cm² (based on dry weight) a) Occurrence of purple coloration during incubation likely due to catechol-metal complexation. b) Representative sample after 20 hours in the exposure chamber

As with the L-dopa experiments, the incubation solutions of MeFP-1 in water began to turn noticeably purple within 10-30 minutes (see Figure 3.3), indicating the formation of dopa-metal complexes in the protein solution⁹,⁵⁰. A purple color, in particular, has been linked to the MeFP-1-iron(III) complex⁵⁰. No color change has been observed in the case of catechol-iron(II) complexes⁶¹. In all three experiments, the purple color became more intense with time, indicating steadily increasing amounts of metal complexation in the incubation solution. The presence of iron ions in solution is indicative of corrosion, but it is unclear if the presence of the protein accelerates or hinders this corrosion.
In Figures 3.5 and 3.6, anodic sites can be seen forming during the incubation period, when the sample is in contact with the protein solution. In Figure 3.5b, it can clearly be seen that corrosion continuing at these sites after the sample was placed in the exposure chamber. The experiments with MeFP-1 in deionized water showed increasing amounts of iron in solution during the incubation period, based on the observation that the purple color became more intense during that time (Figure 3.4). Sites of active dissolution were also observed on the HY80 surface during incubation, and corrosion can be seen to continue at these sites during exposure (Figure 3.5 and 3.6). These experiments demonstrate that MeFP-1 is not an effective corrosion inhibitor for HY80 in weakly acidic deionized water at the concentrations used.

Figure 3.5: Incubation of a 1.5mg/mL solution of mixed MeFP in deionized water on an epoxy-mounted HY80 sample a) after 35 minutes of incubation followed by rinsing and b) after 20 minutes of exposure in the exposure chamber.
Figure 3.6: Incubation of 1.5mg/mL MeFP-1 in deionized water. a) Protein-treated HY80 sample 40 minutes after the start of incubation. b) Protein-enzyme mixture 1 hour after the addition of the enzyme solution c) Sample before exposure after the excess incubation solution was removed d) Sample after 20 minutes of exposure in the chamber.

3.1.3 MeFP-1 in Borate-Acetate Buffer

During protein purification, acetic acid was used as the solvent of choice for many of the purification steps; it is volatile (allowing for lyophilization of the protein for long-term storage), and its low pH easily dissolves the basic MeFPs while protecting their L-dopa groups from oxidation. MeFPs have been shown to auto-oxidize when exposed to air at pH’s as low as 4.5, and the rate of auto-oxidation greatly increases around pH 8^{64,65}. 

38
Borate is capable of reducing the auto-oxidation of L-dopa at high pH by the formation of a complex, so it was included in the buffer solution\textsuperscript{57}.

Early experiments with different MeFP formulations in this buffer yielded very different results from those done in deionized water. When any MeFPs were incubated in a borate-acetate buffer at pH 7 or above, no color change was observed. In a few cases, corrosion during the incubation period did occur; but in these cases, corrosion would not occur consistently on other samples that were similarly prepared and treated with the same incubation solutions. Several experiments were done with both pure MeFP-1 and mixed MeFPs in borate acetate buffer, but all of them obtained similar results. One of these experiments, which used dried films of pure MeFP-1 at 0.32mg /cm\textsuperscript{2}, is presented (see Table 3.1 and Figures 3.7-3.9).

The variability observed in the borate-acetate buffer was not confined to the protein incubation period, however. Once in the exposure chamber, some protein-treated samples incubated in borate-acetate buffer would last up to thirty days in the exposure chamber without corrosion; however, some buffer controls would also display similar corrosion resistance.

The time that individual samples treated with borate-acetate buffer spent in the exposure chamber before corroding displayed a large degree of variability. Typically, a few buffer-treated samples would corrode within a few days of chamber exposure, while the remainder of the samples would last weeks or up to a month without corroding. The variability in the time until corrosion was first observed was inevitably reflected in the mass loss and image analysis data that was collected (Figure 3.8-3.9).
**Figure 3.7:** Representative samples from an exposure chamber experiment with MeFP-1 dissolved in borate-acetate buffer.

**Table 3.1:** Time (in hours) elapsed in the exposure chamber until the first corrosion was observed for each sample in the borate-acetate buffered MeFP-1 experiment

<table>
<thead>
<tr>
<th>Borate-Acetate Buffer Control</th>
<th>MeFP-1</th>
<th>Enzyme-Treated MeFP-1</th>
<th>Untreated Control</th>
<th>Commercial Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>9</td>
<td>0↑</td>
<td>1</td>
<td>72</td>
</tr>
<tr>
<td>26</td>
<td>108</td>
<td>0↑</td>
<td>1</td>
<td>72</td>
</tr>
<tr>
<td>48</td>
<td>156</td>
<td>0↑</td>
<td>1</td>
<td>96</td>
</tr>
<tr>
<td>156</td>
<td>180</td>
<td>204</td>
<td>1</td>
<td>96</td>
</tr>
<tr>
<td>324</td>
<td>720*</td>
<td>720*</td>
<td>1</td>
<td>96</td>
</tr>
<tr>
<td>444</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>617</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>720*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>720*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>720*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Sample did not corrode during the length of the experiment (36 days)*

↑Sample corroded during protein incubation (before being exposed to the chamber)
Figure 3.8: Mass loss of the borate-acetate buffered samples after 30 days in the exposure chamber. Error bars are one standard deviation. ANOVA analysis did not show significant difference between the treatments. The sample size for each treatment is presented in Table 3.1.

Figure 3.9: Image analysis data for the borate-acetate buffered samples after 30 days in the exposure chamber. Error bars are one standard deviation. The sample size for each treatment is presented in Table 3.1. ANOVA analysis did not show significant difference between the treatments at any of the times examined.
Although the corrosion on a few of the samples was limited to one or two smaller locations on the samples’ surface and did not corrode, in general corrosion spread to other areas on the sample surface once it had begun to corrode (see Figure 3.7 and Appendix B). Samples that began to corrode early therefore had a much higher mass loss than those that began to corrode later in the experiment. Therefore, the large degree of variability in the time –to-corrosion explains the equally large amount of variability in the mass loss and image analysis data.

3.1.4 Preliminary Phosphate Buffer Exposure Experiments

Preliminary experiments were conducted that evaluated the suitability of phosphate buffer as a replacement for the borate-acetate buffer. Phosphate buffer is an effective buffer near neutral pH, and it has been used by other research groups with MeFP-1 to inhibit the corrosion of carbon steel with some limited success41.

These samples marked the first exposure chamber experiment in which any protein-treated samples displayed a clear improvement over relevant buffer control samples (in this case the protein was also treated with the crosslinking enzyme). Perhaps more importantly, the buffer controls themselves all began to corrode after one hour in the exposure chamber, so the corrosion resistance of the protein-treated samples can be unambiguously linked to the presence of enzyme-treated protein.

However, it was realized after the experiment that the properties of the air-formed oxide layer formed during the time between sample preparation and the experiment may have affected the performance of the protein-buffer system in the exposure chamber. Specifically, the ‘aged oxide’ samples, which were prepared 14-28 days before the
experiment, performed much better than the ‘fresh oxide’ samples, which were prepared 48 hours before the experiment.

The fresh oxide samples did display some of the same purple coloration as the samples treated with MeFP in deionized water did, although the color was not nearly as intense. Throughout the incubation, small purple aggregates also formed in the incubation solution. These aggregates are the small dark spots that can be seen on the surface of the fresh oxide samples after 1 hour of exposure (Figure 3.10, controls in Figure 3.11)

![Figure 3.10: Images of HY80 treated with protein and enzyme after various periods in the exposure chamber. Samples were treated with pure MeFP-1 in a pH 7.0, 0.05M phosphate buffer for one hour, followed by a one-hour incubation with a crosslinking enzyme.](image)

From these results, it was determined that the status of the oxide layer formed on HY80 was important to the function of MeFP-1 as inhibitors in phosphate buffer. Samples used
in subsequent experiments were stored in heated, dry air (>50% RH) for a uniform amount of time before being exposed in the chamber.

XPS was employed to better understand the differences in the air-formed oxide layer between and a ‘fresh’ oxide sample and an ‘aged’ oxide sample. The data is presented in Table 3.2.

![Image] Figure 3.1: Images of representative HY80 buffer control samples treated with a 0.05M phosphate buffer at pH 7.0 after various periods in the exposure chamber
The large amount of carbon present on the sample is likely due to the contamination the samples experienced while being exposed to the air for several days. The ratio of oxidized iron to unoxidized iron in the sample is not very different between the two samples considering the variability observed, and it is larger for the sample that was aged for less time, which is the reverse of what might be expected. Other than small differences in the bonding of oxygen, the samples appear similar. The kinetics of oxide formation on iron in air at room temperature is logarithmic, so it seems possible that there are no substantial differences in the air formed oxide on HY80 between 2 and 17 days. The variability in the phosphate tests may be explained by other factors, discussed in section 4.2.
3.2 MeFP-1 in Phosphate-Acetate Buffer

The results of phosphate buffer experiments described in the previous section were promising, and replication of those results with a larger sample size and without any variability in the age of the air-formed oxide was desired. However, the batch of pure MeFP-1 used in the preliminary phosphate experiments was only large enough for the samples used in that experiment; to continue experimentation required the purification of more MeFP-1. After the next batch of MeFP-1 was prepared, initial experiments with the protein revealed that this particular batch would not fully dissolve directly into deionized water. To facilitate the dissolution of the protein, it was decided that the MeFP-1 would first be dissolved in a small amount of 0.1M acetic acid, and then mixed with a phosphate buffer. The pH was also changed to 6.0 for these experiments (rather than the 7.0 used in the phosphate experiments) in an attempt to reduce the likelihood that the acetate would passivate the HY80 surface. Images of the exposure chamber samples from this experiment are presented in Appendix B.

From the pictures presented in Figure 3.12 and Appendix B, it can be seen that the controls corrode very quickly or not at all. Some of the control samples began to corrode during the incubation period and continue on to corrode much more severely during the course of the experiment, but those that did not corrode during incubation went on to corrode very little or not at all in the exposure chamber. The protein samples were slightly less variable. None of the protein treated samples (crosslinked or otherwise) corroded during the incubation period, but the non-crosslinked samples all began corroding after 1-4 hours of exposure. Three of the four enzyme-treated samples did not
corrode until after 24 hours in the exposure chamber, which would be noteworthy if their performance wasn’t far outstripped by the controls that did not corrode at all.

\[\text{Figure 3.12:} \] Representative HY80 samples from the experiment described with various treatments. All of the samples are presented in Appendix B.
Table 3.3: Time (in hours) elapsed in the exposure chamber until the first corrosion was observed for each sample in the treatments studied. ANOVA analysis did not show significant difference between the treatments.

<table>
<thead>
<tr>
<th>Buffer Control (0.01M Acetate, 0.045M Phosphate)</th>
<th>Commercial Inhibitor</th>
<th>MeFP-1</th>
<th>Enzyme Treated MeFP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0(^\dagger)</td>
<td>24</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>0(^\dagger)</td>
<td>72</td>
<td>1</td>
<td>72</td>
</tr>
<tr>
<td>0(^\dagger)</td>
<td>72</td>
<td>1</td>
<td>72</td>
</tr>
<tr>
<td>0(^\dagger)</td>
<td>72</td>
<td>4</td>
<td>168(^*)</td>
</tr>
<tr>
<td>1</td>
<td>96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>168(^*)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>168(^*)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>168(^*)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>168(^*)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Sample did not corrode during the length of the experiment (7 days)
\(^{\dagger}\) Sample corroded during protein incubation (before being exposed to the chamber)

The data presented in Table 3.3 and Figures 3.13-14 clearly show that the performance of the MeFP-1 treated samples falls far short of the aged-oxide phosphate samples discussed in the previous section. Furthermore, the time to corrosion data is reminiscent of the variability observed in the borate-acetate experiments. In a manner similar to the borate-acetate experiments, variability in the time until corrosion begins leads to a large variability in the mass loss and image analysis data. The ANOVA analysis did not show any significant differences between any of the treatments in either the time to corrosion, mass loss, or image analysis data, confirming that the variability in these experiments prevents any conclusions regarding the effectiveness of the MeFP-1 from being drawn.
It is worth mentioning that dissolving the MeFP-1 in acetic acid before mixing it with phosphate buffer did not succeed in dissolving all of the protein. A portion of the protein did not dissolve in the acetic acid, and remained undissolved after the addition of the buffer solution to the mixture.

**Figure 3.13**: Mass loss data from the MeFP-1 exposure chamber experiment. Error bars are one standard deviation. One-way ANOVA analysis revealed no significant difference between the treatments. The sample size for each treatment is presented in Table 3.3.
Figure 3.14: Image analysis data from the MeFP-1 exposure chamber experiment. Error bars are one standard deviation. One-way ANOVA analysis revealed no significant difference between the treatments. The sample size for each treatment is presented in Table 3.3.

The MeFP-1 EIS experiment was conducted using the same buffer system and batch of protein that was used in the MeFP-1 exposure experiment. The data is presented in Figure 3.15. Figure 3.15 show that the MeFP-1, enzyme treated or otherwise, did not have an effect on the corrosion of HY80 compared to the control samples.
**Figure 3.15:** EIS data for the MeFP-1 experiment. The cell containing MeFP-1 Sample 1 had a slow leak that went unnoticed, so there is no data after 3 days for that sample (the reference electrode lost contact with the solution). One-way ANOVA analysis revealed no significant difference between the treatments.

All of the samples used for EIS corroded to some degree during the incubation period, unlike the exposure chamber samples, in which some samples displayed a degree of corrosion resistance in the incubation period. Representative samples are shown in Figure 3.16.

**Figure 3.16:** Representative samples of MeFP-1 treatments in the EIS experiment at the end of the incubation period. From right to left: buffer control, MeFP-1, enzyme-treated MeFP-1.
3.3 MeFP-3 in Phosphate Buffer

The methods used in the MeFP-3 experiment more closely followed the example set by the preliminary phosphate buffer experiment that were incubated in 0.05M phosphate buffer at pH 7.0. In fact, the only difference in the incubation conditions between that experiment and the MeFP-3 experiment is the pH, which was lowered to 5.5 for the MeFP-3 and MeFP-5 experiments. This pH was used because it is thought to approximate the pH of the mussel protein secretion. In a separate experiment, sodium periodate (NaIO₄) was used to crosslink the MeFP-3, instead of mushroom tyrosinase.

Pictures of the exposure chamber samples are presented in Figures 3.17-18 as well as in Appendix B. In many respects, the results of this experiment are not dissimilar from the preliminary experiments conducted in phosphate buffer; the controls all corroded quickly upon being placed in chamber (if not during incubation; see Table 3.4), while some of the enzyme-treated protein samples did not corrode during the 7-day exposure period. When corrosion occurred before the incubation period, a grey corrosion product very quickly covered the entire face of the sample. It appears to happen with a similar frequency on both protein-treated samples and buffer controls. Samples that corroded in this fashion during the incubation period corroded more quickly and severely than other samples. The same blue-grey corrosion product was observed on the control samples that did not corrode during incubation, but rather than forming over the entire sample the corrosion product formed on the bottom half of the sample and along the edges of the masking tape. These areas would be more likely to still be in contact with phosphate solution, so the initial corrosion on all of the control samples is somewhat similar in appearance and linked to the presence of the phosphate buffer solution.
Figure 3.17: Representative samples from the MeFP-3 exposure chamber experiment

Neither the periodate-treated protein samples or the periodate controls corroded during the incubation period. The corrosion progressed very similarly for both the controls and
the protein-treated samples. For both treatments, corrosion began around the edge of the exposed region of the samples and spread to a majority of the sample by day 4. Both treatments also contained samples on which the corrosion was confined to a few very small locations.

**Table 3.4:** Time (in hours) elapsed in the exposure chamber until the first corrosion was observed for each sample in the treatments studied. Letters indicate significant differences calculated determined via the Tukey-Kramer method.

<table>
<thead>
<tr>
<th>Phosphate Buffer Control</th>
<th>MeFP-3</th>
<th>Enzyme Treated MeFP-3</th>
<th>NaIO₄ Treated Phosphate Buffer</th>
<th>NaIO₄ Treated MeFP-3</th>
<th>Commercial Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0⁺</td>
<td>0⁺</td>
<td>0⁺</td>
<td>1</td>
<td>1</td>
<td>72</td>
</tr>
<tr>
<td>0⁺</td>
<td>0⁺</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>72</td>
</tr>
<tr>
<td>1</td>
<td>0⁺</td>
<td>144</td>
<td>24</td>
<td>1</td>
<td>72</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>168*</td>
<td>24</td>
<td>4</td>
<td>96</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>168*</td>
<td>72</td>
<td>10</td>
<td>96</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>168*</td>
<td>72</td>
<td>72</td>
<td>96</td>
</tr>
</tbody>
</table>

* Sample did not corrode during the length of the experiment (7 days)
† Sample corroded during protein incubation (before being exposed to the chamber)
**Figure 3.19:** Mass loss after seven days of exposure for the MeFP-3 experiments. Error bars are one standard deviation. Letters indicate significant differences calculated determined via the Tukey-Kramer method. The sample size for each treatment is presented in Table 3.4.

**Figure 3.20:** Image analysis data from the MeFP-3 exposure chamber experiments. Error bars are one standard deviation. Letters indicate significant differences calculated determined via the Tukey-Kramer method. The sample size for each treatment is presented in Table 3.4.
The mass loss and image analysis data is presented in Figures 3.19-20. It can be seen that even when the time to corrosion data is tightly clustered, there is still a considerable amount of variation in the mass loss and image analysis data. Despite this fact, there is a significant difference between the phosphate buffer controls and the enzyme-treated MeFP-3 samples in both time to corrosion and mass loss, but not in the image analysis data. However, the inconsistent occurrence of corrosion during the incubation period with both MeFP-3 treated samples and buffer control samples indicates that MeFP-3 may not be capable of inhibiting corrosion consistently.

**Figure 3.21**: EIS data comparing the performance of enzyme-treated MeFP-3 with six 0.05M phosphate buffer controls.
The EIS experiments presented in Figures 3.21-22 show that MeFP-3 has no effect on the corrosion of HY80 in the electrolyte solution, either at the time of the first measurement after three hours of immersion, or after 1-5 days of immersion. These results are similar to the EIS results obtained from MeFP-1, which also showed that the protein solution had no effect. ANOVA analysis showed that MeFP-3 did not have a statistically significant effect on $R_p$ compared with the buffer controls at any point in time, regardless of crosslinking.

### 3.4 MeFP-5 in Phosphate Buffer

The MeFP-5 experiments followed the same incubation protocol as the MeFP-3 experiments. A shortage of available MeFP-5 meant that one of the treatments would have to have a reduced sample size. In most of the previous experiments, the enzyme-treated protein outperformed the normal protein samples, so priority was given to the

![Figure 3.22: EIS data comparing the performance of non-crosslinked MeFP-3 with six 0.05M phosphate buffer controls.](image-url)
enzyme-treated protein treatment condition. Due to delays in protein purification, the MeFP-5 samples were aged for 24 days in dry air before being treated and exposed, instead of 16 days as in the MeFP-3 experiments.

![Image](image_url)

**Figure 3.23:** Representative samples from the MeFP-5 experiments

Pictures of the exposure chamber samples are presented in Appendix B, with representative samples shown in Figure 3.23. The phosphate buffer controls in this experiment looked very similar to the phosphate buffer controls used in the previous experiment. This was expected; the only difference between the two sets of controls in these experiments is the slightly longer time the samples used for the MeFP-5 experiments were stored before use. A gray corrosion product tended to form on the buffer control samples in the more wetted areas as with the previous set of controls, and all of the control samples corroded within an hour of exposure. The time to corrosion, mass loss, and image analysis data are presented in Table 3.5 and Figures 3.24-25.
Table 3.5: Time (in hours) elapsed in the exposure chamber until the first corrosion was observed for each sample in the MeFP-5 experiment. Letters indicate significant differences calculated determined via the Tukey-Kramer method.

<table>
<thead>
<tr>
<th>Phosphate Buffer</th>
<th>MeFP-5</th>
<th>Enzyme Treated MeFP-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0†</td>
<td>1</td>
<td>120</td>
</tr>
<tr>
<td>1</td>
<td>168*</td>
<td>168*</td>
</tr>
<tr>
<td>1</td>
<td>168*</td>
<td>168*</td>
</tr>
<tr>
<td>1</td>
<td>168*</td>
<td>168*</td>
</tr>
<tr>
<td>1</td>
<td>168*</td>
<td>168*</td>
</tr>
<tr>
<td>1</td>
<td>168*</td>
<td>168*</td>
</tr>
</tbody>
</table>

* Sample did not corrode during the length of the experiment (7 days)
† Sample corroded during protein incubation

Figure 3.24: Image analysis data for the MeFP-5 exposure experiment. Error bars are one standard deviation. Letters indicate significant differences calculated determined via the Tukey-Kramer method. ANOVA analysis did not show significant differences between the treatments at 1 day. The sample size for each treatment is presented in Table 3.5.
Figure 3.25: Mass loss data for the MeFP-5 exposure experiment. Error bars are one standard deviation. Letters indicate significant differences calculated determined via the Tukey-Kramer method. The sample size for each treatment is presented in Table 3.5.

Of the non-crosslinked MeFP-5 samples, one of them began corroding around the edges of the sample after one hour in the exposure chamber, while the other did not corrode for the length of the experiment. There is therefore no significant difference between the non-crosslinked MeFP-5 and the buffer controls in time-to-corrosion, but significant differences were observed in the imageJ and mass loss data. Because of the small sample size, it is not prudent to draw conclusions regarding the noncrosslinked MeFP-5 treatment, despite the results of the statistical analyses.

The same cannot be said for the enzyme-treated MeFP-5. After treatment with enzyme, a thick, white buildup could be seen on the samples’ surface. Once in the exposure chamber, none of the samples corroded, except for one sample which showed a tiny spot of corrosion after five days in the chamber. The spot represented 0.1% of the sample’s exposed surface by the end of the experiment. A significant increase in time to corrosion
and percent inhibition was observed between the enzyme-treated MeFP-5 samples and the phosphate-buffered controls. A significant decrease in mass loss was also observed between those two treatments.

Despite the success of the enzyme-treated MeFP-5 samples in the exposure chamber, neither crosslinked or non-crosslinked MeFP-5 films had an effect on the corrosion of HY80 immersed in 0.1M NaCl, as measured by EIS (see Figures 3.26-27). ANOVA analysis showed that both crosslinked and noncrosslinked MeFP-5 did not have a statistically significant effect on $R_p$ compared with the buffer controls at each time of the time periods that were examined.

**Figure 3.26:** EIS data comparing the performance of HY80 treated with MeFP-5 with six 0.05M phosphate buffer controls.
Figure 3.27: EIS data comparing the performance of enzyme-treated MeFP-5 on HY80 with six 0.05M phosphate buffer controls.
4.1 Passivation in the Borate-Acetate Buffer

Both acetate and borate are known to passivate iron under certain conditions\(^{67,69-72}\). In the case of acetate, the exact mechanism is disputed, but dissolved oxygen, acetate concentration, and pH all affect the tendency for acetate solutions to promote the formation of a passive layer on iron\(^{70,73,67}\). In general, a higher pH acetate solution promotes the formation of a passive layer, while the effect of acetate concentration is more complicated\(^{67}\). Like acetate, borate passivity occurs at neutral to basic pH and is dependent on factors such as dissolved oxygen and the presence of iron ions in solution\(^{70,74}\). A considerable amount of work has been directed toward identifying the structure of the passive film that forms on iron in a mildly alkaline borate buffer, although most of these efforts have studied films grown via anodic polarization rather than ones formed with immersion techniques\(^{71}\). In fact, most of the recent literature on both borate and acetate passivity have been electrochemical studies on well-defined substrates (such as pure iron) not used in real-life applications under well-controlled electrochemical conditions. While these studies have yielded insights into the structure or formation mechanisms of these coatings, they have little direct relevance to this work.
That being said, given that the pH range is within the range in which acetate is capable of passivating iron\textsuperscript{67}, it seems likely that the HY80 samples are being passivated by the borate-acetate buffer. Since increased pH increases the tendency of acetate to passivate iron, a lack of passivation would explain the stark difference in corrosion observed between borate-acetate samples incubated at pH 6.5 and 7.0 (See Figure 4.1).

![Figure 4.1: Representative HY80 samples treated with MeFP in borate-acetate buffers at two different pHs after 24 hours: a) At pH 6.5 b) at pH 7.0](image)

Under normal conditions, it seems reasonable to expect that similar untreated steel samples exposed to similar conditions would begin corroding at approximately the same time and corrode at roughly the same rate. Likewise, a coating of uniform thickness applied consistently to similar samples might be expected to begin corroding at similar times, since the underlying processes that control the failure of such coatings, such as water ingress\textsuperscript{2}, are also understood to be directly related to the exposure conditions, which were well controlled in this experiment. This proved to be true in the case of the commercial inhibitor samples, which began corroding within 24 hours of one another, but it did not prove true for any of the samples treated with borate-acetate buffer, including the protein-treated samples. The commercial inhibitor was prepared in a solution of DI water, which did not contain any acetate or borate, so this variability can be attributed to
the presence of borate and acetate ions. If the unreliable corrosion resistance demonstrated by some of the borate-acetate treated samples is a result of the formation of passive layers, then the time elapsed before the onset of corrosion would be controlled by the processes that dictate the breakdown or dissolution of those passive layers.

Once formed, passive layers exist in a dynamic equilibrium between the reactions that govern film dissolution and film growth\textsuperscript{75}. Tests with untreated HY80 (Table 3.1) show that HY80 corrodes very quickly upon being placed in the exposure chamber, which suggests that untreated HY80 does not form a protective passive layer in air. Therefore, it seems reasonable to assume that after the original solution of borate-acetate buffer has washed away in the exposure chamber, any reactions that may contribute to the growth of the passive layer are probably insignificant. However, if the environment of the exposure chamber (40°C, 100% relative humidity) was aggressively promoting the dissolution of the oxide layer formed during incubation, then it might be expected that all of the samples would have corroded after as much as 30 days of exposure. Therefore, it seems likely that the passive layers formed during incubation persist in the exposure chamber in a metastable state. Studies of pre-passivated iron in acid media may be analogous to this condition. In these circumstances, iron oxide layers are thermodynamically unstable, and the protection of the underlying metal is contingent on the slowness of the dissolution kinetics of the oxide\textsuperscript{36,76}.

If it is assumed that the passive layer is relatively stable, and uniform dissolution of the entire passive layer is not the cause of the corrosion that was observed, then the corrosion that did occur must then be explained by the localized failure of the passive layer. In this respect, the corrosion observed in these samples is similar to pitting. However, it is
important to note that in the case of the experiments discussed here, there is a lack of aggressive ions such as chloride, since deionized water was used to create the humid fog in the exposure chamber. Most of the theories that have been put forward to describe pit initiation involve the action of the chloride ion, but many authors have noted that underlying metallurgical defects such as grain boundaries, inclusions, and precipitates can cause the disruption of passive films\textsuperscript{34,75,76}. Because of this, it seems possible that localized breakdown phenomena similar in some respects to pitting corrosion might be occurring.

The occurrence of pitting is not always easy to predict. In fact, pitting events are often considered to be random events rather than the results of deterministic processes for the purposes of modeling\textsuperscript{77}. Studies investigating the pitting potential of 304L stainless steel at different scan rates observed that there is a large degree of variation regarding the exact potential at which pitting begins. The variation in these values was normally distributed, suggesting natural variation\textsuperscript{78}. A property known as the induction time has also been investigated. Induction time is defined as the time required for a stable pit to form following a sudden increase in potential into the pitting range, or following the injection of chloride into a nonaggressive solution\textsuperscript{77}. The induction time has also been found to be normally distributed, and it can be predicted with some accuracy based on the point-defect model of pit initiation\textsuperscript{79}.

If the onset of pitting corrosion as a result of localized breakdown of a passive film can be accurately modeled as a random event then it is possible that some of the variation seen in the borate-acetate buffer is a result of similar stochastic processes. After early experiments with borate-acetate buffer displayed large degrees of variation in corrosion
susceptibility, every effort was made to ensure that the samples used were thoroughly cleaned and as uniform as possible. Therefore, the large degree of variability observed in both the controls and the protein-treated samples in the borate acetate experiments is suspected to be an unavoidable consequence of the passivation of the HY80 and the exposure conditions used.

Another factor that likely contributes to the large error observed in all of the exposure chamber experiments is the small physical size of the samples used. For all the experiments discussed, the exposed area was only 0.5cm². The small area was necessitated by the shortage of pure protein, which precluded the use of larger samples, such as the 3x5 inch panels which are more commonly used for exposure chamber experiments. Corrosion tended to occur preferentially at the sample edge, since the interface between the electrochemical tape and the exposed area was constantly wetted. The use of smaller samples meant that the area wetted due to the presence of the masking tape accounted for a larger portion of the total area of the sample than it would have on a larger sample, potentially leading to more erratic behavior in the exposure chamber.

4.2 Corrosion and Passivation in Phosphate Buffer

Phosphate buffer is an effective buffer around neutral pH, and it has been frequently used in biochemistry in the past\(^\text{80}\). But while the effects of other common biological buffers on metal surfaces have not necessarily been the subject of much research, the effects of phosphate solutions on ferrous metals have been widely studied, albeit in the context of industrial phosphate coatings.
Phosphate conversion coatings have been used for corrosion inhibition for over a century\textsuperscript{81}. They are primarily used to increase the corrosion resistance of a subsequently applied coating, but the porous nature of these coatings also helps to promote coating adhesion\textsuperscript{82}. By themselves, the coatings are usually too porous to offer much corrosion protection\textsuperscript{82}. The most common industrial phosphate coatings are based on solutions of zinc, manganese or iron phosphate at a relatively low pH provided by phosphoric acid, but there is a method of phosphate coating that uses solutions containing only sodium or potassium phosphate solutions. This process is of interest because the solution used is similar to ours; it typically consists of phosphate solution with a pH ranging from 3.5 to 6.0 and a phosphate concentration of 0.015-0.15M\textsuperscript{82}. It is referred to as a “light weight iron phosphating” process or an “alkali metal phosphating” process.

The alkali metal phosphating process has been reviewed in a few texts\textsuperscript{81,82}. The process can be broken down into two stages: a corrosion reaction followed by the redeposition of relatively insoluble iron phosphates. The first stage is under cathodic control; it can proceed very slowly if the pH is high and no dissolved oxygen is present. Oxidizing agents are usually added to accelerate the cathodic portion of the corrosion reaction. After sufficient dissolution of iron, iron phosphates will precipitate on the metal’s surface due to their low solubility in water. As a result, the deposition of the iron phosphate is contingent on nucleation phenomena. Nucleation of iron phosphate does not usually occur until the phosphate solution is supersaturated with iron. When nucleation does occur, iron phosphate rapidly precipitates from the solution. The iron phosphate coating that is formed from this process is variously described as being iridescent, blue or grey in color depending on the thickness of the coating. The composition of the coating is not
fully agreed upon, and likely varies with the process used; different researchers have found different compositions for the phosphate coating deposited by alkali metal phosphating processes. Rausch\textsuperscript{82} mentions three studies, which found coatings on steel to be composed of (respectively): Fe$_3$(PO$_4$)$_2$•8H$_2$O (viviante) and some Fe$_3$O$_4$, FePO$_4$ and Fe(OH)$_2$, or FeHPO$_4$•H$_2$O and Fe$_2$O$_3$.

The alkali metal phosphating process is not perfectly analogous to the conditions used in the phosphate buffered experiments discussed in this work; differences include the high temperature used in industrial conditions (60-70°C), the oxidizing agents used to speed the corrosion reaction, and the presence of surfactants and other additives. However, the solutions are of similar pH and phosphate content, and the grey or bluish-grey corrosion product that forms on many of the samples during incubation matches the description of the coating deposited by the alkali metal phosphating process. Furthermore, the rapid deposition of iron phosphate following the formation of nuclei in a supersaturated iron phosphate solution would explain the sudden onset and uniformity of the corrosion product that formed in the instances when the phosphate-buffered samples corroded during the incubation period.

Although the mechanism of the alkali metal phosphating process is based on the corrosion of the metal substrate, phosphate can passivate iron under certain conditions. Early work by Pryor and Cohen\textsuperscript{83} using iron coupons immersed in phosphate solutions demonstrated that phosphate solutions were capable of passivating iron in the presence of dissolved oxygen, provided the pH was above pH 7.25 and the concentration of phosphate was sufficiently high (between about 0.05M and 0.1M phosphate). At pH values and concentrations lower than required for passivity, aerated solutions of
phosphate corroded at a faster initial rate than in deaerated solutions, due to the availability of dissolved oxygen for cathodic reactions. However, in aerated solution, after a few days, corrosion rates began to slow significantly. In deaerated solutions, corrosion proceeded at a steady rate. In both cases, the corrosion was under cathodic control. The corrosion rates measured by Pryor and Cohen for iron immersed in phosphate solutions were much higher than the corrosion rates of samples immersed in deionized water, except in the conditions when passivity of the iron was achieved. However, these rates were measured via mass loss after 1-5 days, so they may not be relevant to the phosphate incubations in this work, since the HY80 samples were exposed to phosphate solution for less than an hour. The presence of an air-formed oxide, in particular, may reduce the rate of initial corrosion.

In another paper, the influence of surface condition on the composition of the passive layer formed at high pH was studied. Pure iron samples with varying surface treatment were passivated by exposure to a 0.1M phosphate solution at pH 8.5. The surface treatments for the iron samples were: freshly abraded iron with 3/0 emery paper, iron dipped in 0.2N HCl and quickly rinsed to remove the air-formed oxide, and a sample abraded and left in dry air for 24 hours. The importance of controlling the dissolved oxygen in the phosphate solution was stressed; the author stated that thorough shaking of all phosphate solutions was necessary to avoid “very erratic results”. The composition of the coating was determined using various methods. It was found that destroying the air-formed oxide layer resulted in a larger amount of phosphate in the oxide layer, which the authors attributed to increased corrosion occurring during the formation of the passive
layer. Data collected more recently suggests that the passive layer formed at basic pH contains mainly vivianite in addition to some iron oxides\textsuperscript{85}.

Pryor and Cohen also reported some effects due to air-formed surface oxides\textsuperscript{84}. In a deaerated 0.1M phosphate solution containing 10ppm chloride at pH 8.5, steel samples with different surface treatments were placed in contact with a reference electrode and had their $E_{corr}$ monitored. The $E_{corr}$ of freshly abraded samples decreased quickly and monotonically, while the $E_{corr}$ of samples exposed to air for 24 hours prior to experimentation remained noble for a few hours before suddenly decreasing. Based on their observations, the authors attributed this phenomenon to metastable passivity. Similar effects have been observed in 20mM phosphate buffer at pH 7.0\textsuperscript{86}.

A few recent studies have shown that phosphate ions at neutral pH are capable of reducing the dissolution of bulk iron oxides resulting from reaction with dissolved H$_2$S or dissolution-promoting chelators such as EDTA\textsuperscript{40,49}. Phosphate’s ability to inhibit the dissolution of iron oxides has been attributed the formation of stable binuclear surface complexes that bridge two metal atoms, the presence of which has been hinted at or confirmed through infrared spectroscopy\textsuperscript{40,87,88}. The ability of phosphate to form dissolution-inhibiting complexes is recognized to be pH dependent; phosphate inhibits dissolution on iron oxides only at higher pH (above 5-7, depending on the conditions)\textsuperscript{40,49}. It seems likely that this property is related to the tendency of phosphate to induce passivity at high pH.

The fact that HY80 samples treated with phosphate buffer consistently corrode shortly after being placed in the exposure chamber shows that the phosphate buffer has a clear
advantage over the acetate-containing buffers for the purposes of analyzing the protein’s performance. According to Pryor’s data, iron will not passivate at pH 5.5 in 0.1M phosphate buffer, with or without dissolved oxygen\(^{70,83}\). Therefore, the MeFP 3 and 5 experiments are in the pH region where corrosion takes place, and this corrosion eventually resulted in what appears to be the deposition of some form of iron phosphate in the case of the control samples. However, there was clearly some variability in the corrosion rate between samples having the same treatment, because some samples corroded to the point where what is presumed to be a form of iron phosphate was deposited during the sample incubation period, while others in the same treatment did not. (see Figure 4.2)

\[\text{Figure 4.2: Possible iron phosphate deposition on control samples incubated in 0.05M phosphate buffer after 1 hour in the exposure chamber}\]
Samples that were fully covered by corrosion product (presumably iron phosphate) by the end of the incubation period corroded more over the course of the experiment than samples that showed no visible corrosion upon entering the chamber. This variability was reflected in the image analysis and the mass loss data.

However, it is not known exactly what the effect the pH 5.5 phosphate buffer had on the air-formed oxide layer on HY80. If the phosphate buffer reduces the dissolution rate of the oxide layer under these conditions, then the air formed oxide may have a degree of metastability of the type observed by Pryor and Cohen at pH 8.0 in chloride solution. If this is indeed the case, it might explain the variability observed in the corrosion during the incubation period observed in the experiments done in 0.05M phosphate buffer. It might also explain the difference in corrosion during the incubation period between protein dissolved in DI water and protein dissolved in phosphate buffer.

The amount of dissolved oxygen in the buffer solutions used was also not controlled. This may have caused some variability between different treatments in the susceptibility of samples to corrosion during the incubation period, since the corrosion is likely under cathodic control. The treatments were also not applied at a consistent temperature, since the protein solutions were stored in the refrigerator for short periods before the experiment to reduce protein oxidation. Oxygen is more soluble in cold water, therefore the protein solutions might have been subjected to a slightly harsher corrosion environment during the incubation period.

Variations in the amount of dissolved oxygen may explain the variability observed between the fresh and aged oxide samples in the preliminary phosphate tests (Section
3.14). The XPS data did not reveal any differences in the oxide surface between samples aged in the laboratory for 2 days and 17 days. However, although the same batch of protein was dissolved under the same buffer conditions, the fresh oxide samples were incubated with the protein-buffer solution on a different day with different solutions. Therefore, is possible that there were differences in the dissolved oxygen present in the solution. This may account for the differences in the corrosion rate observed on samples with similar surface oxides.

4.3 Corrosion of HY80 Samples Treated with MeFP Solutions in Deionized Water

In the experiments in which HY80 samples were treated with DI water, unambiguous corrosion was observed. From Appendix A, it can be seen that much of the protein used in these experiments contained between 5-14% L-dopa. It is clear that the protein by itself is not capable of preventing the corrosion of HY80. It is likely that the corrosion was encouraged to some degree by the lower pH that was measured for this solution (4.0-5.0). It is also possible that the surface complex formed by protein-bound L-dopa promotes the dissolution of the metal in the same way low-molecular weight catechols have been shown to. If this were the case, then some of the visible iron-catechol complexes present in the solution may have been formed as a result of the dissolution of surface complexes from the metal oxide. However, in the borate-acetate, phosphate-acetate, and phosphate buffers, no purple coloration was seen in the incubation solution, indicating that no catechol-iron complexes were formed within the limits of visual detection.
One explanation for this lack of complex formation is that these buffers promote the formation of a stable passive layer, or otherwise help maintain the air-formed oxide in a meta-stable state. If this were true, then if the protein-bound L-dopa promoted the dissolution of these iron oxides, then some evidence of purple complex formation might be expected in solutions buffered with phosphate, borate, or acetate. This was not observed. This does not prove that peptide-bound dopa does not promote the dissolution of the iron oxide film formed on HY80, but it may indicate that the majority of the iron in solution present in the deionized water experiments is present as a result of anodic dissolution not involving the dissolution of a surface complex involving L-dopa. While it could be the case that the catechol-iron complexes that are formed in these experiments are dissolution-promoting and the phosphate, borate, and acetate ions are out-competing them for surface sites, this seems unlikely due to the high stability complexes of iron-catechol complexes.

The stability of the catechol-iron surface complex formed by MeFP-1 on HY80 is not known at this or any pH, and it cannot be determined from the present data. However, there are reasons to believe that the MeFP-1 is incapable of uniformly covering the metal surface. Reasons for this spotty coverage might include steric issues as well as the probability of repulsion forces dominating between proteins that are overwhelmingly positively charged. This might be particularly true in the case of the protein samples that were used for the deionized water experiments, as they were composed of 40-50% arginine in some cases (see Appendix A). To further complicate matters, iron oxide surfaces have a pH$_{pzc}$ of 9.8-10, so the predominance of positively charged amino acids does not bode well for adsorption in this case$^{48}$. This means that at the pH ranges
were investigated, the net charge of the oxide was positive, and therefore it cannot be expected that positively charged residues would lend themselves to greater adsorption in this case.

4.4 MeFP-1 in Phosphate-Acetate Buffer

The addition of small amounts of acetate to the buffer greatly affected the susceptibility of the controls to corrosion. Although many of the control samples corroded during the incubation period, the ones that did not proved to be nearly impervious to corrosion over the course of the experiment. Although the pH of the buffer was chosen specifically so that acetate passivation would not be a factor in those experiments as it was in the borate-acetate buffer, it did not prevent the passivation of a few of the control samples. It seems as if there may have been a synergistic effect that increased the range of pH in which either phosphate or acetate was capable of passivating iron. Similar to the borate-acetate experiments, the resistance of the controls that did not corrode during incubation to further corrosion makes it impossible to attribute any corrosion resistance observed in the MeFP-1 treated samples to the effect of the protein.

The non-crosslinked protein performed poorly, in terms of corrosion inhibition. Although no corrosion occurred during incubation, small white flecks of aggregated protein can be observed on the surface of the metal after the incubation period. In the crosslinked samples, these aggregates appear larger and have a brown coloration. Because the protein did not fully dissolve, these aggregates were present in the protein solution when it was applied, but they may have increased in size due to the oxidation of the protein, either via oxidation due to exposure to dissolved oxygen, or due to crosslinking.
Table 4.1 compares the amino acid content of the protein used in this experiment with another batch of MeFP-1 that was used for the preliminary experiments with MeFP-1 in phosphate buffer, as well as the composition of the protein originally reported by Waite et al. 92.

**Table 4.1:** Various amino acid compositions obtained for MeFP-1, in mol%. Nonpolar residues are black, polar residues are blue, residues having a charge at pH 5.5 are red. The Asn/Asp and Gln/Glu ratios are not known.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Earlier Batch of MeFP-1</th>
<th>MeFP-1 used in this experiment</th>
<th>Original reported sequence of MeFP-1 92</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>1.4</td>
<td>4.6</td>
<td>8.1</td>
</tr>
<tr>
<td>Gly</td>
<td>3.5</td>
<td>5.1</td>
<td>3.2</td>
</tr>
<tr>
<td>Ile</td>
<td>1.0</td>
<td>2.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Leu</td>
<td>0.6</td>
<td>3.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Met</td>
<td>0.1</td>
<td>3.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Phe</td>
<td>0.3</td>
<td>3.8</td>
<td>0.1</td>
</tr>
<tr>
<td>Pro</td>
<td>1.3</td>
<td>4.3</td>
<td>8.1</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.2</td>
<td>6.4</td>
<td>6.6</td>
</tr>
<tr>
<td>Val</td>
<td>1.2</td>
<td>3.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Cys</td>
<td>0.1</td>
<td>5.3</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Dopa</strong></td>
<td><strong>18.1</strong></td>
<td><strong>3.2</strong></td>
<td><strong>10.6</strong></td>
</tr>
<tr>
<td>Hyp</td>
<td>1.2</td>
<td>2.7</td>
<td>13.1</td>
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<tr>
<td>Ser</td>
<td>2.7</td>
<td>4.6</td>
<td>10.2</td>
</tr>
<tr>
<td>Thr</td>
<td>3.2</td>
<td>4.0</td>
<td>11.7</td>
</tr>
<tr>
<td>Arg</td>
<td>53.5</td>
<td>19.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Asp</td>
<td>1.4</td>
<td>4.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Glu</td>
<td>2.9</td>
<td>5.0</td>
<td>0.9</td>
</tr>
<tr>
<td>His</td>
<td>2.1</td>
<td>9.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Lys</td>
<td>4.3</td>
<td>5.2</td>
<td>21.0</td>
</tr>
</tbody>
</table>
The amino acid content of the MeFP-1 used in the phosphate-acetate buffered MeFP-1 experiments probably explains the poor solubility and reduced effectiveness of the protein. The MeFP-1 used in this experiment has a much larger amount of hydrophobic functional groups, such as leucine, methionine, phenylalanine, and others. Furthermore, the critically important functional group L-dopa is only present at 3.2%. It is not uncommon for protein composition to vary from organism to organism or from population to population, and Dopa groups in particular are vulnerable to oxidation during the course of protein purification. This data stresses the importance of determining the amino acid content of protein used for research.

It is also possible that the variability in the amino acid content of MeFP-1 purified for these experiments might arise from differences in the methods used for purification. In this case, the batch used in the MeFP-1 experiments had been purified from mixed MeFP samples from the S-400 column, rather than coming straight from the G-200 column as the earlier batches had (see Figure 2.1). Therefore, the protein may have degraded to some extent during the extra time it spent at room temperature while being separated on the size-exclusion columns.

It’s worth noting that even with very little L-dopa content; the crosslinked MeFP-1 protein appears to have successfully formed a barrier film that may have marginally improved the resistance of the HY80 samples to corrosion, compared with the non-crosslinked MeFP-1. However, due to the variability of the buffer-only controls, there is no way of knowing how much of this corrosion resistance is a result of the MeFP-1 film and how much is a result of the properties of the phosphate or acetate ions.
4.5 MeFP-3 in Phosphate Buffer

It is presumed that the corrosion that occurs during the incubation period on some samples is the precipitation of iron phosphate. For this to occur, iron ions must first supersaturate the solution directly above the surface. Therefore, if the MeFP-3 is capable of inhibiting corrosion by maintaining the pre-existing air-formed oxide layer, then no corrosion (iron phosphate precipitation or otherwise) during the incubation period would be expected. This was not observed; one third of the MeFP-3 treated samples showed the formation of a grey corrosion product, the same proportion as the controls. Therefore, there is no evidence that the MeFP-3 is acting as an inhibitor during the incubation period.

However, after the incubation period is over, the MeFP-3 treatment does seem to have an effect, at least in the case of the crosslinked samples. Of these samples, only one experienced extensive corrosion that began after the incubation period. This indicates that the crosslinked MeFP-3 film is effective at inhibiting corrosion in the exposure chamber, even if the non-crosslinked MeFP-3 is ineffective at preventing corrosion in the incubation solution. If the MeFP-3 is acting as an inhibitor by stabilizing the oxide layer, then it may be that its effectiveness is determined to some degree by the state of the oxide layer at the beginning of the experiment. Mass loss data collected by Pryor\textsuperscript{90} seems to indicate that phosphate buffer is a more corrosive medium to steel in general than DI water at pH’s less than 7, at least on the timescale of 1-5 days. However, there is a possibility that the air-formed oxide of the sample is metastable in shorter timer periods, which might explain the variability of the corrosion during the incubation period. In either case, it may be that if the oxide layer is stabilized such that it does not corrode
during the incubation period, a coating of crosslinked protein may stabilize it further such that it can survive a considerable length of time in the exposure chamber, as was observed.

**Table 4.2:** The amino acid composition obtained for MeFP-3, in mol\%, compared to a sequence reported by Waite et. al. Standards that would allow the detection of hydroxyarginine were not run in this experiment. The Asn/Asp and Gln/Glu ratios are not known.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>MeFP-3 used in this experiment</th>
<th>Reported sequence of MeFP-3&lt;sup&gt;28&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>3.7</td>
<td>2</td>
</tr>
<tr>
<td>Gly</td>
<td>5.0</td>
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</tr>
<tr>
<td>Ile</td>
<td>1.9</td>
<td>0</td>
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<tr>
<td>Leu</td>
<td>7.5</td>
<td>0</td>
</tr>
<tr>
<td>Met</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>Phe</td>
<td>1.2</td>
<td>0</td>
</tr>
<tr>
<td>Pro</td>
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<td>6</td>
</tr>
<tr>
<td>Trp</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.3</td>
<td>2</td>
</tr>
<tr>
<td>Val</td>
<td>1.8</td>
<td>0</td>
</tr>
<tr>
<td>Cys</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td><strong>Dopa</strong></td>
<td><strong>1.9</strong></td>
<td><strong>20</strong></td>
</tr>
<tr>
<td>Hyp</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>Ser</td>
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<td>1</td>
</tr>
<tr>
<td>Thr</td>
<td>3.3</td>
<td>0</td>
</tr>
<tr>
<td>Arg</td>
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<td>6</td>
</tr>
<tr>
<td>H-Arg</td>
<td>ND&lt;sup&gt;†&lt;/sup&gt;</td>
<td>12</td>
</tr>
<tr>
<td>Asp</td>
<td>6.0</td>
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<tr>
<td>Glu</td>
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<td>0</td>
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<tr>
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<td>1</td>
</tr>
<tr>
<td>Lys</td>
<td>7.4</td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>†</sup>Not Determined
The amino acid composition determined for the MeFP-3 used in this work is presented in Table 4.2. Although many different variants of MeFP-3 have been reported by Waite et al., none of them are similar to the observed composition\textsuperscript{28}. The large concentration of arginine (45\%) is particularly noteworthy, as is the large reduction in dopa compared with the reported sequence (2\% vs. 20\%). The latter phenomenon is most readily explained by oxidation of the L-Dopa residues during the protein purification process. The acid-urea gel for this batch of MeFP-3 is presented in Appendix B. It shows that the MeFP-3 used in these experiments is slightly degraded, and a very faint band of MeFP-2, another MeFP protein, can be observed.

There is even less L dopa present in the MeFP-3 samples than there was in the MeFP-1 samples. This may explain to an extent the inability of the MeFP-3 to inhibit corrosion during the incubation period of the protein.

The MeFP-3 used in this experiment contained a larger amount of different varieties of hydrophobic residues than might have been expected based in the literature. This might be seen as a bad thing, since too much hydrophobicity would reduce the solubility of the protein, and hydrophobic amino acids would be among the least likely to form strong adhesive bonds due to their tendency to interact chiefly with each other in water. However, this same tendency would be beneficial in a coating, since a more hydrophobic coating would prevent the ingress of water. Therefore, the greater hydrophobicity of the MeFP-3 used in this experiment compared to MeFP-3 variants previously reported may have improved its performance after crosslinking had occurred.
It is slightly surprising that the crosslinked protein was able to prevent corrosion as well as it did over the long term, considering these issues. In particular, the small amount of L-dopa present in the protein would seem to be particularly problematic. However, many of the crosslinking mechanisms for catechol-containing polymers that have been proposed involve the reaction between a quinone-intermediate and another amino acid. Amino acids that have been variously proposed to be involved in crosslinking reactions include lysine, histidine, cystine and other L-dopa groups, so the protein may not require a large amount of L-dopa in its composition to crosslink effectively\textsuperscript{19}.

Metal-mediated crosslinking is also an important potential crosslinking mechanism, but it would be more reliant on large amounts of L-dopa in the protein composition, since metal-mediated crosslinking would involve 2-4 L-Dopa residues depending on the type of complexation involved, rather than 1-2 L-dopa groups needed for other forms of crosslinking\textsuperscript{19}. If it were a predominant mode of crosslinking, a color change would also be observed. The results of the MeFP-1 in DI water suggest that complexation of iron by MeFP-1 does not, by itself, lead to an effective coating. It might be that intramolecular crosslinking predominated in this particular circumstance, or it might be that metal-mediated crosslinking of MeFP-1 does not form an effective coating for corrosion inhibition in general.

\textbf{4.5.1 Experiments with NaIO\textsubscript{4}-treated MeFP-3}

In preliminary experiments (not shown), 20mM periodate added to a phosphate buffer appeared to promote the formation of a passive layer on HY80 in 0.05M phosphate buffer at pH 5.5. As a powerful oxidizing agent, periodate likely has a similar effect to dissolved
oxygen in phosphate buffers, promoting passivation or possibly corrosion depending on the circumstances. Compared to the phosphate buffer controls without periodate, the controls treated with periodate have a greater corrosion resistance, with no obvious corrosion occurring during the incubation period. The MeFP-3 treated samples appeared similar to the controls. Only a small amount of periodate was added (0.096mM), so it is possible that the protein was not fully crosslinked, since the periodate may also have been consumed in reactions with the metal oxide layer. This might explain why the periodate-treated samples did not show the same corrosion resistance as the enzyme-treated samples, despite the lack of corrosion during incubation. Differences have also been observed in the structure of periodate-treated MeFP films versus enzyme-treated films, with enzyme-treated thin films having a greater mass as measured by quartz-crystal microbalance. This difference was explained by co-adsorption of the enzyme during the crosslinking process, resulting in a thicker layer. Therefore, it is possible that enzyme-treated protein might form a more effective barrier film than chemically crosslinked MeFP. The effect on corrosion inhibition of periodate-treated mussel protein films vs. that of enzyme treated films will likely remain difficult to quantify on HY80 due to the former’s tendency to promote the passivation of that substrate.

4.6 MeFP-5 in Phosphate Buffer

Unlike MeFP-3, none of the MeFP-5 samples, crosslinked or otherwise, corroded during the incubation period. This indicates that the MeFP-5 inhibited the corrosion of the HY80 during the incubation period. MeFP-5 is also unique among the proteins tested in that for at least one sample the non-crosslinked protein was also effective.
The amino acid composition of the MeFP-5 used as determined from the amino acid analysis is presented in Table 4.3. Like the MeFP-3 that was used, there is much less L-dopa present than would be expected based on the literature, and this is likely due to the oxidation of the L-dopa groups during purification. However, of the pure proteins that were tested in phosphate buffer or phosphate-acetate buffer, the MeFP-5 that was used has the largest amount of L-dopa (3.6%) and the smallest amount of hydrophobic amino acids.
Table 4.3: The amino acid composition obtained for MeFP-5, in mol%, compared to a sequence reported by Waite and Qin. Standards that would allow the detection of phosphoserine were not run. The Asn/Asp and Gln/Glu ratios are not known.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>MeFP-5 used in this experiment</th>
<th>Reported sequence of MeFP-5&lt;sup&gt;32&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>3.1</td>
<td>4.7</td>
</tr>
<tr>
<td>Gly</td>
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<sup>†</sup>Not Determined

The increased amount of L-dopa may explain why the exposure chamber samples treated with MeFP-5 did not corrode during the incubation period while the MeFP-3 treated samples did. In many respects, the protein is similar to the MeFP-3 that was used, except that it has almost double the amount of L-dopa. In addition, the MeFP-1 used in the
phosphate-acetate buffered experiments contained almost as much L-dopa as a percentage of its total composition as the MeFP-5, and the MeFP-1 treated exposure chamber samples also did not corrode during the incubation period. However, since dissolved oxygen was not well controlled between different treatments, it cannot be definitively concluded that the increased L-dopa content is responsible for the reduced incidence of corrosion during the incubation period. After the incubation period was concluded, the crosslinked MeFP-3 samples that did not corrode during incubation were just as effective as the crosslinked MeFP-5 samples, since for both treatments almost all of the enzyme treated samples also did not corrode during the 7-day exposure period. Two-sided t-tests showed no significant difference between the crosslinked MeFP-3 and MeFP-5 treatments in time to corrosion, mass loss, or percent inhibition, so it cannot be said that crosslinked MeFP-3 performs significantly better than MeFP-5.

The hypothesized reason for the effectiveness of the crosslinked protein is that the crosslinking of proteins increases the thickness and density of the protein film, creating a more impermeable coating. This impermeability of such a coating would impede the transport of aggressive ions and charged species through the film and thus reduce corrosion. It is also possible that the crosslinked layers might be restricting the transport of phosphate ions away from the metal surface. If the phosphate ions are contributing to the stability of the air formed oxide by the formation of surface complexes, then they too might contribute to the significant increase in corrosion resistance that was observed, at least until the phosphate diffuses away from the metal surface.
4.7 EIS Experiments

In the MeFP-1 experiment, the protein treated samples did have a larger $R_p$ than the control samples in the EIS experiments. This slight difference in $R_p$ was maintained over the 5-day experiment in the case of the enzyme-treated MeFP-1, but not for the uncrosslinked MeFP-1. However, this difference was not large enough to have any real physical significance. Considering the poor solubility of the protein and its hydrophobic amino acid composition, and the variable performance of the exposure chamber samples, these results are not surprising.

In all of the EIS experiments, a greater portion of the protein-treated samples corroded during incubation. The reason for this is not known, but it is possible that small air bubbles present around the seal of the O-ring might have provided the solution near the metal surface with an increased supply of oxygen, encouraging corrosion via the formation of an oxygen concentration cell. It is also possible that the air-formed oxide may have been damaged by the motion of the O-ring when the sample was clamped into the cell.

In the MeFP-3 EIS samples, four of the six samples showed corrosion during incubation, while all of the MeFP-1 and 5 samples corroded to some extent in the incubation solution. In the case of the MeFP-5 experiments, the incubation solution turned a light purple color. However, samples that did not corrode during incubation in the MeFP-3 experiment did not have higher $R_p$ values compared to other samples in the treatment. This suggests that whatever happened to the HY80 samples after they came into contact
with the electrolyte solution may have had more of an effect on the polarization resistance of the samples than the state of the samples beforehand.

None of the differences between crosslinked and non-crosslinked samples in the exposure chamber manifested themselves in the EIS data. The similarity of all of the protein-treated samples to the control samples is likely due to the more corrosive nature of the bulk electrolyte solution compared to the exposure chamber experiments. The chloride ion, in particular, is detrimental to oxide layers as well as coatings\textsuperscript{34}. Even the commercial inhibitor, despite its consistent effectiveness in the exposure chamber, did not maintain a significantly higher $R_p$ than untreated HY80 controls (data not shown). Therefore, the conditions used in the EIS experiment might be too severe to meaningfully test temporary corrosion inhibitors of this type on low-alloy steels.

The EIS data obtained from these experiments are not far removed from the results described in section 1.2.5 by Zhang which involved crosslinked MeFP-1 on carbon steel incubated at pH 9.0\textsuperscript{42}. While they did observe a small increase in $R_p$, the increase was not large enough nor did it persist long enough to have a significant impact on the corrosion rate. Zhang et. al. used commercially available MeFP-1 and did not verify its composition, so it is not known what the L-dopa content of the protein used in their experiments was. In the one circumstance in which MeFP-1 has been linked to an increase in $R_p$ of multiple orders of magnitude, the substrate was pure aluminum, the buffer was 0.05M sodium phosphate at pH 7.0, and the protein used was 17.4\% dopa\textsuperscript{15}. The performance of the protein in this circumstance is most likely made possible by the increased dopa content, which would allow for an increased amount of crosslinks to be formed and thus presumably a thicker, more impermeable coating. However, the effect of
other variables such as the specific surface complex formed between catechol and aluminum oxide may have also played a critical role in determining the effectiveness of the coating formed by MeFP-1 in that case.

4.8 Conclusions

The following conclusions relating to the hypotheses that were listed in Section 1 can be drawn from the experiments discussed:

**Hypothesis 1:** *Mytilus edulis* foot proteins 1, 3 and 5 inhibit the flash rusting of HY80 steel in the exposure chamber.

**Conclusions:** The ability of the mussel proteins tested to inhibit the corrosion of HY80 is strongly dependent on the solution that the proteins are dissolved in. By themselves, both MeFP-1 and a MeFP mix (composed of 5% and 14% L-dopa, respectively) failed to prevent the corrosion of HY80 in an incubation solution containing the protein dissolved in deionized water. In the borate-acetate buffer, no effect related to the MeFP-1 could be indisputably discerned, due to the passivation of the HY80 by that buffer solution as evidenced by the controls. Similar results were obtained in a buffer containing 0.045M phosphate and 0.01M acetate at pH 6.0. However, control samples treated with only 0.05M phosphate buffer at pH 5.5 did not show any evidence of passivation in the exposure chamber conditions tested. In this buffer, treatment with crosslinked MeFP-3 prevented the flash rusting of HY80 in the exposure chamber for the entirety of the seven-day experiment on some samples, but other MeFP-3 treated samples (crosslinked and otherwise) corroded during the incubation period. The cause of this variability is suspected to be a result of the natural variation in the protectiveness of the air formed
oxide, which may be stabilized to a degree by the formation of surface complexes with either the phosphate buffer or L-Dopa groups present in the protein. Despite this variability, crosslinked MeFP-3 significantly reduced the mass loss of HY80 samples over the 7-day exposure chamber experiment. Crosslinked MeFP-5 was more successful; the mass loss, time to corrosion, and image analysis data all showed that MeFP-5 significantly inhibited the flash rusting of HY80 steel in 0.05M phosphate buffer at pH 5.5. In many respects, the inhibition was similar to that observed with MeFP-3, except in no instance did the samples treated with MeFP-5 corrode during incubation. It was speculated that this might be due to corrosion inhibition resulting from the increased amount of L-dopa present in the composition of that batch of MeFP-5, but other explanations cannot be ruled out, such as the potential for variability in the amount of dissolved oxygen initially present in the buffer solution.

**Hypothesis 2:** Crosslinking will increase the amount of corrosion resistance that the MeFPs confer to HY80 steel by increasing the thickness and water-resistance of the MeFP films, resulting in better performance in the exposure chamber and higher polarization resistance as measured by Electrochemical Impedance Spectroscopy (EIS).

**Conclusions:** Crosslinking convincingly improved the resistance of MeFP-3 and MeFP-5 films to corrosion in the exposure chamber. In these cases, only MeFP-treated samples that were also treated with crosslinking enzyme resulted in a significant decrease in mass loss or a significant rise in time to corrosion or percent inhibition compared to a buffer-only control. However, the EIS data did not indicate that the presence of any of the MeFPs had a statistically or physically significant effect at any point in the experiment. This was true of all protein-treated samples, even those that were also treated with
crosslinking enzyme. These results were attributed to the more severe corrosion environment of the electrochemical tests, as well as the increased frequency of corrosion during the incubation period for the EIS samples. A commercial inhibitor which successfully prevented flash rusting in the exposure chamber also did not show any increase in Rp compared with an untreated control, so it was determined that this method might not be ideal for testing flash rust inhibitors of this type.

**Hypothesis 3:** Differences in the performance of the MeFPs will be directly related to the L-dopa content in the protein, as verified by amino acid analysis data.

**Conclusions:** Comparisons between the effectiveness of different batches and molecular weights of MeFPs are difficult if not impossible to make, due to the many changes that were made to the methods between different experiments, and the use of passivating buffers such as acetate, which may have obscured the analysis of the protein’s performance in some cases. Even direct comparison between the MeFP-3 and MeFP-5 tests, which used the same methods and the same buffer solution, is made more complicated by the fact that no effort was made to control the concentration of dissolved oxygen in the buffer solutions that were used. However, in these two experiments, greater corrosion inhibition was observed during the incubation period with the crosslinked MeFP-5 samples compared to the MeFP-3 samples. As the L-dopa content of the MeFP-5 was nearly double that of MeFP-3 (3.5% vs. 1.9%), this difference could be attributed to the higher L-dopa content of the MeFP-5. While this might appear to confirm the hypothesis that increases in the L-dopa content of a protein result in more enhanced corrosion inhibition, more experiments would be necessary to confirm this.
4.8.1 Future Work

Future work is being planned that will investigate the effect of synthetic analogs to MeFP-5. The use of synthetic analogs will eliminate the time required to extract and purify natural proteins, as well as eliminating the variability inherent in natural products. Furthermore, synthetic analogs will be available in much larger quantities than mussel protein, allowing for physically larger samples as well as larger samples sizes. Future experiments investigating the effect of corrosion inhibitors in phosphate buffer should make efforts to control the amount of dissolved oxygen available in solution as well as the presence and/or thickness of air-formed oxides.
REFERENCES


(14) Zhang, F. The Mussel Adhesive Protein (Mefp-1) — A Green Corrosion Inhibitor,
KTH Royal Institute of Technology, 2013.


Appendix A

Protein Purity and Composition
Experiment: MeFP in Deionized Water (Section 3.1.2, Figure 3.4)

Protein: Pure MeFP-1

Total mass of protein detected per mg dry weight: 0.231 mg/mg dry weight

Acid-Urea PAGE gel information: 1µL of a 1 mg/mL protein solution was loaded

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<td>Lys</td>
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**Experiment:** MeFP in Deionized Water (Section 3.1.2, Figure 3.5)

**Protein:** Mixed MeFP

**Total mass of protein detected per mg dry weight:** 0.488 mg/mg dry weight

**Acid-Urea PAGE gel information:** 10µL of a 1 mg/mL protein solution was loaded.
Experiment: MeFP in Deionized Water (Section 3.1.2, Figure 3.6)

Protein: Pure MeFP-1

Acid-Urea PAGE gel information: 10µL of a 1 mg/mL protein solution was loaded

No amino acid analysis data for this protein
**Experiment:** MeFP-1 in Borate-Acetate Buffer (Section 3.1.3)

**Protein:** Pure MeFP-1

**Acid-Urea PAGE gel information:** 10µL of a 1 mg/mL protein solution was loaded.

No amino acid analysis data for this protein.
Experiment: Preliminary Phosphate Buffer Exposure Experiments (Section 3.1.4)

Protein: Pure MeFP-1

Total mass of protein detected per mg dry weight: 0.425 mg/mg dry weight

Acid-Urea PAGE gel information: 10µL of a 1 mg/mL protein solution was loaded

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Experiment: MeFP-1 in Phosphate-Acetate Buffer (Section 3.2)

Protein: Pure MeFP-1

Total mass of protein detected per mg dry weight: 0.465 mg/mg dry weight

Acid-Urea PAGE gel information: 20µL of a 1 mg/mL protein solution was loaded
Experiment: MeFP-3 Experiments (Section 3.3)

Protein: Pure MeFP-3

Total mass of protein detected per mg dry weight: 0.259 mg/mg dry weight

Acid-Urea PAGE gel information: A 1 mg/mL protein solution was loaded

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Experiment: MeFP-5 Experiments (Section 3.4)

Protein: Pure MeFP-5

Total mass of protein detected per mg dry weight: 0.243 mg/mg dry weight

Acid-Urea PAGE gel information: 10µL of a 1 mg/mL protein solution was loaded

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Appendix B

Exposure Chamber Images
MeFP-1 in Borate-Acetate Buffer (Section 3.13)

**Substrate:** HY80 samples with 1200 grit surface finish

**Buffer:** 0.437M acetate, 0.025M, borate pH 7.0

Before Exposure
MeFP-1 in Borate-Acetate Buffer (Section 3.13)

**Substrate:** HY80 samples with 1200 grit surface finish

**Buffer:** 0.437M acetate, 0.025M, borate pH 7.0

10 days

MeFP-1

Enzyme-treated MeFP-1

Buffer
MeFP-1 in Borate-Acetate Buffer (Section 3.13)

**Substrate:** HY80 samples with 1200 grit surface finish

**Buffer:** 0.437M acetate, 0.025M, borate pH 7.0

20 days
MeFP-1 in Borate-Acetate Buffer (Section 3.13)

**Substrate:** HY80 samples with 1200 grit surface finish

**Buffer:** 0.437M acetate, 0.025M, borate pH 7.0

30 days
MeFP-1 in Phosphate-Acetate Buffer (Section 3.2)

**Substrate:** HY80 samples with 600 grit surface finish

**Buffer:** 0.045M Phosphate, 0.01M Acetate, pH 6.0

**Before Exposure**

![Before Exposure Images](image-url)
MeFP-1 in Phosphate-Acetate Buffer (Section 3.2)

Substrate: HY80 samples with 600 grit surface finish

Buffer: 0.045M Phosphate, 0.01M Acetate, pH 6.0

1 day
MeFP-1 in Phosphate-Acetate Buffer (Section 3.2)

**Substrate:** HY80 samples with 600 grit surface finish

**Buffer:** 0.045M Phosphate, 0.01M Acetate, pH 6.0

4 days

![MeFP-1 images]

MeFP-1

![Enzyme-treated MeFP-1 images]

Enzyme-treated MeFP-1

![Commercial Inhibitor images]

Commercial Inhibitor

![Buffer images]

Buffer
MeFP-1 in Phosphate-Acetate Buffer (Section 3.2)

**Substrate:** HY80 samples with 600 grit surface finish

**Buffer:** 0.045M Phosphate, 0.01M Acetate, pH 6.0

7 days

MeFP-1

Enzyme-treated MeFP-1

Commercial Inhibitor

Buffer
MeFP-3 in Phosphate-Acetate Buffer (Section 3.3)

**Substrate:** HY80 samples with 600 grit surface finish

**Buffer:** 0.05M Phosphate, pH 5.5

**Before Exposure**

MeFP-3  Enzyme-treated MeFP-3  Buffer  Commercial Inhibitor
MeFP-3 in Phosphate-Acetate Buffer (Section 3.3)

**Substrate:** HY80 samples with 600 grit surface finish

**Buffer:** 0.05M Phosphate, pH 5.5

1 day

![MeFP-3](image)

![Enzyme-treated MeFP-3](image)

![Buffer](image)

![Commercial Inhibitor](image)
MeFP-3 in Phosphate-Acetate Buffer (Section 3.3)

**Substrate:** HY80 samples with 600 grit surface finish

**Buffer:** 0.05M Phosphate, pH 5.5

4 days

MeFP-3

Enzyme-treated MeFP-3

Buffer

Commercial Inhibitor
MeFP-3 in Phosphate-Acetate Buffer (Section 3.3)

**Substrate:** HY80 samples with 600 grit surface finish

**Buffer:** 0.05M Phosphate, pH 5.5

**7 days**

- **MeFP-3**
- **Enzyme-treated MeFP-3**
- **Buffer**
- **Commercial Inhibitor**
MeFP-3 in Phosphate-Acetate Buffer (Section 3.3)

**Substrate:** HY80 samples with 600 grit surface finish

**Buffer:** 0.05M Phosphate, pH 5.5

**Before Exposure**

![Images of MeFP-3 before exposure](Image)

- NaIO$_4$-treated MeFP-3
- NaIO$_4$-treated Buffer 1 day

![Images of MeFP-3 after exposure](Image)

- NaIO$_4$-treated MeFP-3
- NaIO$_4$-treated Buffer 1 day
MeFP-3 in Phosphate-Acetate Buffer (Section 3.3)

**Substrate:** HY80 samples with 600 grit surface finish

**Buffer:** 0.05M Phosphate, pH 5.5

---

**4 days**

NaIO$_4$-treated MeFP-3

NaIO$_4$-treated Buffer

---

**7 days**

NaIO$_4$-treated MeFP-3

NaIO$_4$-treated Buffer
MeFP-5 in Phosphate-Acetate Buffer (Section 3.4)

**Substrate:** HY80 samples with 600 grit surface finish

**Buffer:** 0.05M Phosphate, pH 5.5

**Before Exposure**

MeFP-5

Enzyme-treated MeFP-5

Buffer Controls

Commercial Inhibitor
MeFP-5 in Phosphate-Acetate Buffer (Section 3.4)

**Substrate:** HY80 samples with 600 grit surface finish

**Buffer:** 0.05M Phosphate, pH 5.5

1 day

MeFP-5

Enzyme-treated MeFP-5

Buffer Controls

Commercial Inhibitor
MeFP-5 in Phosphate-Acetate Buffer (Section 3.4)

**Substrate:** HY80 samples with 600 grit surface finish

**Buffer:** 0.05M Phosphate, pH 5.5

4 days

**MeFP-5**

**Enzyme-treated MeFP-5**

**Buffer Controls**

**Commercial Inhibitor**
MeFP-5 in Phosphate-Acetate Buffer (Section 3.4)

**Substrate:** HY80 samples with 600 grit surface finish

**Buffer:** 0.05M Phosphate, pH 5.5

7 days

MeFP-5

Enzyme-treated MeFP-5

Buffer Controls

Commercial Inhibitor