Methoxypyrazine removal in grape juice: Development of a removal system using odorant and pheromone binding proteins coupled with bentonite fining

By

Eric Fabian Humes, B.Sc. in Biology

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Brock University

St. Catharines, Ontario, Canada

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ABSTRACT

Multicoloured Asian Lady Beetles (MALB) and 7-spot Lady Beetles that infect vineyards can secrete alkyl-methoxypyrazines when they are processed with the grapes, resulting in wines containing a taint. The main methoxypyrazine associated with this taint is 3-isopropyl-2-methoxypyrazine (IPMP). The wines are described as having aroma and flavours of peanut butter, peanut shells, asparagus and earthy which collectively, have become known as "ladybug taint". To date, there are no known fining agents used commercially added to juice or wine that are effective in removing this taint.

The goal of this project was to use previously identified proteins with an ability to bind to methoxypyrazines at low pH, and subsequently develop a binding assay to test the ability of these proteins to bind to and remove methoxypyrazines from grape juice. The piglet odorant binding protein (plOBP) and mouse major urinary protein (mMUP) were identified, cloned and expressed in the *Pichia pastoris* expression system. Protein expression was induced using methanol and the proteins were subsequently purified from the induction media using anion exchange chromatography. The purified proteins were freeze-dried and rehydrated prior to use in the methoxypyrazine removal assay. The expression and purification system resulted in yields of approximately 78% of purified plOBP and 62% of purified mMUP from expression to rehydration. Purified protein values were 87 mg of purified plOPB per litre of induction media and 19 mg of purified mMUP per litre of induction medium. In order to test the ability of the protein to bind to the MPs, an MP removal assay was developed. In the assay, the purified protein is incubated with either IPMP or 3-isobutyl-2-methoxypyrazine (IBMP) for two hours in either buffer or grape juice. Bentonite is then used to capture the protein-MP complex and the bentonite-protein-MP complex is then removed from solution by filtration. Residual MP is

measured in solution following the MP removal assay and compared to that in the starting solution by Gas Chromatography Mass Spectrometry (GC/MS). GC/MS results indicated that the mMUP was capable of removing IBMP and IPMP from 300 ng/L in buffer pH 4.0, buffer pH 3.5 and Riesling Juice pH 3.5 down to the limit of quantification of the instrument, which is 6ng/L and 2ng/L for IBMP and IPMP, respectively. The results for the plOBP showed that although it could remove some IBMP, it was only approximately 50-70 ng/L more than bentonite treatment followed by filtration, resulting in approximately 100 ng/L of the MPs being left in solution. pIOBP was not able to remove IPMP in buffer pH 3.5 using this system above that removed by bentonite alone. As well, the pIOBP was not able to remove any additional MPs from Chardonnay juice pH 3.5 above that already removed by the bentonite and filtration alone. The mouse MUP was shown to be a better candidate protein for removal of MPs from juice using this system.

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1. Introduction:

1.1 Background

Wine aroma is formed by the intricate balance of several hundred compounds (Perez-Preito *et al.*, 2003). These volatile compounds are grouped into categories, such as "fruity" or "floral", based on common perceptions. One important class of wine aromas is termed "vegetative/herbaceous", which includes descriptors such as "bell pepper", "asparagus" and "grassy". Vegetative aromas are critical constituents of wine aroma. They are elicited by two chemical classes of compounds, the aliphatic carbonyls and the 3-alkyl-2-methoxypyrazines (MPs) (Blake *et al.*, 2009). These compound classes vary in chemical structure, aroma quality and relative importance in grapes and wines. The MPs that contribute to greenness in wines from grape-derived MPs are nitrogen-containing heterocyclic ring structures, with alkyl side groups, known as the 3-alkyl-2-methoxypyrazines.

MPs are important constituents of wine flavour because of special sensory attributes. MPs can cause a perceived decrease in intensity, or "masking", of other aromas and also can define the varietal character in certain wines and wine-styles (Kosteridis *et al.*, 1999; Lacey *et al.*, 1991). MPs present in nature possess an intense and unique aroma-potency; they are interesting or attractive at low concentrations, but at slightly higher levels become very characteristic and repellent. The same holds true in wine, as elevated MPs can be overpowering and negative to wine aroma. Their significance is additionally evidenced by their prevalence throughout the natural world. MPs occur in a wide survey of raw vegetables and other food products (Sala *et al.*, 2002), are produced by microorganisms (Pickering *et al.*, 2008) and as

pheromone compounds by insects (Al Abassi *et al.*, 1998) and plant species (Dunlevy *et al.*, 2009) likely as warning compounds to repel predators.

1.2 The Problem

Grape pests cause economic loss to growers and wineries by reducing crop, damaging fruit and vines, reducing fruit quality and tainting wines (Ker and Carter, 2004). Two new pests have been identified as causing problems in Niagara wines: in 2001 an insect, known as the Multicoloured Asian Lady Beetle (MALB) caused losses in the millions to the Niagara wine industry (Pickering and Lin, 2006B; OGWRSC Annual Report 2002-2008); and in 2007 an insect, known as the 7-spot lady beetle, which over past years has become a growing concern especially with respect to problems in vineyards (Ker and Carter, 2004; Botesatu *et. al.,* 2012). These insects are not a pest that causes direct fruit injury, or affects vine health or growth, but compromises crop quality after the harvest when processed along with the grapes (Ker and Carter, 2004; Botesatu *et. al.,* 2012).

When the lady beetle is experiencing stress, a reflex bleed response occurs in the lady beetles, releasing their haemolymph (Al Abassi *et al.*, 1998). Haemolymph is composed of several volatile chemicals whose roles include serving as a warning cue to indicate danger to other ladybeetles. However, when lady beetle haemolymph is released into grape juice, one particular chemical identified in the haemolymph, IPMP, can taint the wine (Pickering *et al.*, 2004). In a study by Pickering *et al*. (2005) where both chemical and sensory trials were run, it was concluded that IPMP is the principle compound responsible for the taint, known as lady bug taint. The IPMP concentrations that have been found in the tainted wines have been on average

between 7.7-32.7 ng/L (Pickering *et al.*, 2005) above that of the human sensory threshold of IPMP, which is approximately 0.32-2.29 ng/L (Pickering *et al.*, 2007).

1.3 Rationale For This Project

While investigating compounds that bind IPMP, a group of proteins which are naturally present in the mucosa of mammals was identified that bind odorants. The role of these proteins in the mucosa is to bind to volatile odorants and deliver these odorants to membrane receptors in order to trigger odor recognition. This family of proteins are known as lipocalins, with two subclasses of interest, odorant binding proteins (OBP) and Pheromone binding proteins (PBP) (Flower, 1996).

A rat odorant binding protein, OBP-1f, characterized by Briand *et al.* (2000) was found to have high affinity and specificity to IBMP, and was the first protein targeted by the Inglis lab group to test in an MP removal assay (Booram, 2006 unpublished). However, initial experiments were not successful, most likely due to the acidic conditions of the wine/juice matrix, with the pH optima of the protein for binding being neutral (Booram, 2006 unpublished). The OBP-1f is a homodimer when found in a neutral pH (Briand *et al.*, 2000), forming the binding pocket for its ligands. But as pH is lowered to the acidic range, OBP-1f dimer dissociates, and binding to its ligands decreases. It is the two monomers that come together in the dimer form which are needed in order to form the binding pocket (Briand *et al.*, 2000).

Further investigations led to the identification of OBPs and PBPs which are monomers in their native form. In the nasal mucosa of the pig snout, porcine OBP was discovered as a fully functional monomer (Scaloni *et al.*, 2001). Porcine OBP was also identified to be able to bind methoxypyrazines, and it has been concluded that the binding affinity for methoxypyrazines did

not significantly change in an acid pH environment of pH 3.5 (Burova *et al.*, 1999). In the urinary tract of mice, mouse major urinary protein was discovered as a fully functional monomer as well (Lucke *et al.*, 1999). Mouse MUP had been shown in literature to bind to IPMP with a high affinity for the mMUP protein at pH as low as 5.5 (Lucke *et al.*, 1999).

1.4 Project Objective

The thesis project had the main goal of comparing the ability of two proteins, piglet OBP and mouse MUP, to remove methoxypyrazines from grape juice. As part of this study, an MP-removal system was developed using bentonite to capture the protein-MP complex. This was followed by filtration to remove the bentonite-protein-MP complex from solution. The proteins were cloned and expressed in the methylotropic yeast *Pichia pastoris*, purified from the induction media and tested in the MP-removal assay to determine if the proteins could be developed into effective juice/wine fining agents.

1.5 The Project Outline

plOBP and mMUP were cloned and expressed in *P. pastoris* under control of the alcohol oxidase promoter, grown aerobically and induced for target protein expression. The secreted proteins were purified from the induction media using anion exchange chromatography and freeze-dried until tested in the MP-removal system. Confirmation of protein expression and secretion into the induction media was determined using SDS PAGE with visualization of the appropriate band at 18 kDa after methanol induction of the yeast.

To develop the MP-removal system, the correct concentration of bentonite required to remove 45 µM protein in the binding assay over a one hour period was first determined. For the MP-removal system, the freeze-dried protein was first dissolved in buffer or juice to which 300

ng/L of IBMP or IPMP was then added. The protein and MP solution was then allowed to mix for two hours. To capture the soluble protein-MP complex that may have formed, bentonite was then added to the binding reaction and allowed to mix for an additional hour. The insoluble bentonite-protein-MP complex was then removed by centrifugation and filtration through a 0.22 μM filter. The remaining MPs present in solution were measured using GC/MS and compared to the MPs at the start of the binding reactions. Controls were set up with just the MPs added to either buffer or juice, with no further protein or bentonite addition or filtration to determine the starting concentration of MPs in solution. To measure the ability of the filter alone to adsorb MPs from solution, the assay was performed without the addition of protein or bentonite. To measure the ability of bentonite and the filter together to adsorb MPs from solution, the assay was performed without the addition of protein. Residual protein remaining in the reactions after filtration but prior to measuring MPs by GC/MS was determined using the Bradford assay, and protein was visualized in the samples by SDS PAGE stained with Commassie blue.

2. Literature Review

2.1 Introduction to Methoxypyrazines

3-alkyl-2-methoxypyrazines (MPs) contribute important aromas and flavours to *Vitis vinifera* grape varieties and develop naturally during berry growth and maturation (Sala *et al.*, 2002). However, as grapes continue to ripen, MPs decrease in concentration (Ryona *et al*, 2008). The MPs commonly associated in wine are 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP) and 3-*sec*-butyl-2-methoxypyrazine (SBMP) and possess general flavour descriptors of "leafy", "bell pepper", "asparagus" and "nutty" (Sala *et al.*, 2002). Olfactory notes of MPs are a result of their alkyl chains: with smaller chains presenting "roasted", "nutty" aromas and longer side chains exhibiting "green" notes (Lacey *et al.*, 1991). Wine with uncharacteristically strong "vegetative" or "bell pepper" aroma (i.e. Pinot noir or Cabernet franc) is considered of poor quality and associated with unripe grapes. Recently, a further source of MPs has been identified in wine, of insect origin. Specifically, MPs from the Multicoloured Asian Lady Beetle (MALB), *Harmonia axyridis* (Coleoptera: Coccinellidae) have been identified in wine (Pickering *et al.*, 2005; Pickering and Lin, 2006A; Pickering *et al.*, 2007). IBMP tends to be the most abundant natural MP in grapes that carries through into wine and hence is most likely to contribute to wine flavour; whereas IPMP is the principle compound responsible for ladybug taint (LBT) in wines (Pickering *et al.*, 2005). Detection thresholds, the concentration at which an aroma can be detected, and indicating the aromatic-potency of volatile compounds, for wine volatiles are in parts per million (mg/L) or parts per billion (µg/L). However MPs possess distinctly low thresholds at parts per trillion (ng/L) or lower. The human sensory threshold of IPMP, the causal agent of LBT, is 0.32-2.29 ng/L in wine (Pickering *et al.*, 2007). This is where the problem lays, as the IPMP concentration in wine fermented in the

presence of the lady beetle is between 9.7 – 37.7 ng/L (Pickering *et al.*, 2007). Nonetheless, there are a number of viticultural, climatic and oenological factors that must be considered before attributing "green" sensory attributes to LBT.

2.1.1 Effect of Vineyard Management on Grape-Derived MPs

There are numerous viticultural factors that could contribute to "green" sensory attributes. Vine vigor can be affected by the soil's water holding capacity, drainage, and nutritional richness which in turn affects the sunlight available to the grapes and subsequently, the MP content in the berries. Grapes grown in deep, nutrient rich clay soils with high water holding capacity have been tested as having higher "vegetative" characteristics (Noble, 1995). It is suggested that if leaf plucking is not practiced prior to veraison and excessively high leaf layer numbers exist in the vineyard, then sunlight exposure to the fruiting zone is reduced. This results in higher MP concentrations in the fruit and consequently, elevated "green" characteristics. An important viticultural consideration with regards to the impact of MP in grape berries is the pruning regime practiced in the vineyard. A minimal pruning technique leaves a loose and open canopy, increasing sunlight exposure to the fruiting zone and has been shown to produce fruit with eightfold lower MP concentrations than vines subjected to spur pruning (Allen *et al*., 1994; Scheiner *et al.*, 2010). Since the majority of MPs are contained within the skins, pulp and bunch stems, the winery's pressing technique can impact the level of MPs in the finished wine. The amount of press fraction blended into the final wine and extended cap maceration has been suggested by Kosteridis *et al*. (1999) to impact the level of MPs and consequent "green" sensory attributes in the final wines.

2.1.2 Effect of Cool-Climate on MP's

A cool climate is a region that has less than 1390 growing degree days base 10°C of heat per year (Amerine and Winkler, 1944). Grapes grown in cool-climate regions or colder than average vintages tend to display more pronounced "vegetative-herbaceous" characteristics than similar grapes of the same variety grown under warmer climactic conditions (Kosteridis *et al*., 1998). Allen *et al*. (1994) found that lower MP concentrations resulted in wines from warmer vintages than cooler vintages at comparable stages of sugar accumulation, suggesting that MP concentrations decrease more rapidly under increased temperature and sunlight conditions. Recent studies have also suggested that wines from vintages which experienced high humidity levels in the month before veraison were found to exhibit elevated IBMP concentrations (Roujou-de-Boubee *et al*., 2002).

The prevention of elevated MPs from grape sources in wine is the preferred method of control and includes both viticultural practices that alter grape ripening, as the MPs present naturally in grapes are synthesized before the ripening stage of fruit growth and decrease there after (Hashizume *et al.*, 1998). It has been noted that IPMP concentration is less influenced by seasonal climatic factors than IBMP (Belancic and Agosin, 2007). Generally, MP mediation during grape growing involves manipulating fruit light exposure; by minimizing the fruit shading from the vegetative canopy and decreasing planting density (Sala *et al.*, 2002) and varying vine training systems (Sala *et al.*, 2002).

2.2 Effect of MALB on Grape Growing in Niagara Region

With the Niagara Escarpment and Lake Ontario acting as borders, the Niagara region has weather patterns and a climate perfectly suited for growing tender fruit such as grapes (Yee *et al.*, 2002). More than 85% of Ontario's vineyards are found in the Niagara region which allows

for the appropriate growth of a wide range of grape varieties such as Chardonnay, Riesling, Merlot, Cabernet sauvignon, Cabernet franc and Pinot noir. (Yee *et al.*, 2002)

Wine production in Ontario has an estimated economic impact of almost 600 million dollars annually to the province (winecountryontario.ca, 2011), which is why the pest problems are of such concern. Although there are several pest species, there are only a small number of species that are capable of causing extensive economic damage (Ker and Carter, 2004). The Multicoloured Asian Lady Beetles are considered to be one of the pest species that cause a major threat to the quality of wine produced in the area (Pickering *et al.*, 2004).

Exported all the way from Asia, *Harmonia axyridis* belongs to the tribe *Coccinellini* in the insect family *Coccinellidae*, order *Coleoptra* (Koch, 2003). It is known by a variety of names, such as the Asian Lady Beetle, Japanese Lady Beetle, or Halloween Beetle. The beetle is, however, most commonly referred to as the Multicoloured Asian Lady Beetle (MALB) (Ker and Carter, 2004). MALB were originally brought to North America as a biological control agent (Coderre *et al.*, 1995), because of their intense appetite for aphids. *H. axyridis* was a good candidate for biological control for aphid populations, and was first introduced to California in 1916. Over time, its uses have spread to Connecticut, Georgia, Louisiana, Maryland, Washington D.C., Delaware, Maine, Mississippi, Ohio, Pennsylvania, and North Carolin (Koch, 2003). The first occurrence in Ontario was found in the Niagara region in the late summer of 2001 (Koch, 2003).

The common name, Multicoloured Asian Lady Beetle, represents how the colouration and the spotting of the beetle can vary. The head may be black or yellow, or both; the area behind the head, known as the pronotum, is yellow with black markings forming an M-shape.

The body itself is oval shaped and domed or convex in appearance, with the wing covers ranging in colours from yellow-orange to red with the number of spots varying from 19 to zero (Koch, 2003).

Figure 2.1 Multicoloured Asian Lady Beetle, with varying number of spots. Picture taken by Ryan Brewster, KCMS applied Research and Consulting INC, 2003**.**

Adult *H. axyridis* are capable of consuming 15 to 65 aphids per day, while their larvae can consume between 90 and 370 during its development (Koch, 2003). In 2000, the central regions of the United States had reports of massive outbreaks of a new pest of field crops, the soybean aphid. It was 2001 when reports of this new pest hitting southern Ontario surfaced. With the hot dry spring of that year, Ontario offered ideal conditions to provide a great source of food for the aphids. With a massive outbreak in aphid populations, *H. axyridis* multiplied dramatically (Ker and Carter, 2004), raising a number of concerns. They were reported by businesses and homeowners as the insects seemed to be causing a number of nuisances ranging from large aggregations on the side of buildings to foul odors and stains, and even the occasional

biting incidents. One of the biggest concerns was the realization that this new pest was also present in vineyards (Ker and Carter, 2004).

Since 2001, the number of sightings and reports about the presence of Lady Beetles being in vineyards has increased, with as many as 20 to 50 beetles on one cluster (Martinson, 2002). One winemaker reported having very few lady beetles in the vineyard until 3 days prior to harvest, when they arrived in swarms (Martinson, 2002). The reasoning behind this late autumn swarming is because the soy has been cropped so there is a drop in the number of soybean aphids, the food source for MALB; this promotes the migration of the beetles to vineyards, as they are in search of food. *H. axyridis*, which hibernate over the winter, are only able to survive by feeding off of their fat stores (Ker and Carter, 2004).

When MALB are inadvertently incorporated with grapes at harvest, elevated MP concentrations are found, along with development of an off-flavor coined 'ladybug taint' (LBT) (Pickering *et al.*, 2004; Pickering and Lin, 2006B). IPMP is the primary compound identified for LBT (Pickering *et al.*, 2005; Pickering and Lin, 2006A) although IBMP (Pickering *et al.*, 2005; Pickering, unpublished data) and SBMP (Pickering, unpublished data) have also been identified at lower concentrations in some MALB-affected wine. LBT has significantly devalued Ontario grapes and wine since the 2001 vintage (Pickering and Lin, 2006B; OGWRSC Annual Report 2002-2008*)*. Subsequently, MALB has been listed as a priority pest, and one of the greatest threats to the industry this decade (OGWRSC Annual Report 2002-2008). The industry has instigated a zero tolerance policy for Lady Beetles in grapes intended for juice and wine production, with subsequent substantial economic losses to grape growers (Booram, 2006 unpublished).

2.3 Methods for MP Removal

During wine fermentation many new volatiles are produced and lost, however, MPs are predominantly stable even after fermentation (Roujou de Boubee *et al.*, 2002). Grape IBMP content does increase after one day of contact with skins in red wine fermentation (Roujou de Boubee *et al.*, 2002), unlike IPMP and SBMP, confirming that IBMP is present in grape skins in greater amounts and that skin contact or maceration is the main oenological processing stage where extraction occurs and not before. Maceration is the winemaking process where the phenolic materials of the grape – tannins, colouring agents (anthocyanins) and flavour compounds – are leached from the grape skins, seeds and stems into the must, it is here that the ladybugs which are present in the grapes are introduced to the must and the tainting of the juice occurs as the heamolymph is introduced into the must (Pickering *et al., 2004).* For extraction of MPs from Lady Beetles, the main stage of extraction is during the fermentation stage where the fermenting must is in contact with the Lady Beetles (Pickering *et al.,* 2004). Little extraction occurs during the harvest or destemming/crushing stages (Pickering *et al.*, 2004). MP content is substantially reduced with pre-fermentation practices of settling white juice either naturally or with the assistance of bentonite to promote removal of heavier matter for IBMP (Roujou de Boubee *et al.*, 2002) and IPMP (Kosteridis *et al.*, 2008). Unfortunately these practices are not always appropriate or feasible, given winemaking constraints and wine style limitations.

Classical fining agents have been tested to see their effects on the Lady Beetle tainted wines (Pickering *et al.*, 2006). A fining agent is a compound that is added to wine and then subsequently removed from the wine in order to improve the wine's appearance, remove any off flavours and prevent any instability that may develop (Morris and Main, 1995). In 2006, Pickering *et al.* performed a study where multiple different fining agents were tested in order to

try and remove ladybug taint. The fining agents that were used were: bentonite, activated charcoal, oak chips, deodorized oak as well as light treatments in the UV and visible range to try to chemically breakdown the MPs. Each wine was analyzed before and after treatment by GC/MS for IPMP as well as by a sensory panel for MALB taint. Activated charcoal was noted for being the most capable in its ability to reduce IPMP in white wine. In the case of red wines, the most successful fining agent was different than that of white wine, in this case the fining agent was deodorized oak (Pickering *et al.*, 2006). Unfortunately this did not bring the IPMP down to a concentration below its aroma and flavour thresholds. Oak chips were able to reduce the intensity of the taint in both red and white wines, although it was not a cure to the problem, only a masking effect (Pickering *et al.*, 2006).

Following the hypothesis that yeast cells may metabolize MPs as a nitrogen source, a subsequent study tested various commercial yeast strains for the ability to alter wine MP content. The study revealed that most strains had no effect on changing the IPMP concentration in the wine but one strain actually increased the concentration (Pickering *et al.*, 2008).

IPMP is stable in wine and does not degrade over time (Blake *et al.*, 2009). Several fining agents have been tested in attempts to remove IPMP from wine, however no fining agents have been identified that are able to reduce IPMP from wine to below sensory detection threshold nor have any treatments been identified that can degrade IPMP in wine in order to reduce the IPMP concentration (Pickering *et al.*, 2006).

It became apparent that new methods and strategies would need to be investigated in order to determine methods to reduce the presence of MPs in the wine. General grape-derived MP mediation during grape growing involves manipulating fruit light exposure, by minimizing

the fruit shading from the vegetative canopy by non-irritation of vines, decreasing planting density, and varying vine training systems (Sala *et al.*, 2002). Justin Scheiner *et al.* 2010, reported that one hundred percent of the leaf removal on day 10 and day 40 after anthesis, resulted in the greatest reduction in MP concentration in the fruit, this was a new thought in canopy manipulation. In terms of insect-derived MPs, various strategies for lady beetle exclusion from vineyards have been used, including pesticide sprays (Ker and Carter, 2004). However due to pre-harvest intervals (the amount of time after a spray treatment that one may harvest) and the late season influx of *Harmonia axyridis* (HA), pest control using sprays can be difficult requiring spray application days before harvest. Lady beetle-derived MPs are imparted to wine only after ladybugs are included in the first oenological processing stage (crushing/destemming) and not before (Pickering *et al.*, 2007). As stated, post-fermentation traditional oenological techniques used to remove taints and alter aromas proved to not be successful in reducing the MP concentration below the threshold. Thus other pre- and post-fermentation practices need to be investigated.

Initial studies by Pickering *et al.* (2007) showed that the elevated levels of IPMP were found when there were 3 beetles per kilogram of grapes. This information led to providing a baseline target for the reduction of MALB density to avoid the development of LBT. Recommendations were made to industry not to exceed 400 beetles per tonne of grapes, a conservative estimate. This led wineries to investigate other options of reducing lady beetle numbers in harvested fruit, such as using shaker tables as a method to separate the grapes from the beetles. However this is a time-consuming process only applicable for hand harvested fruit and not applicable to large processors. Because of the devastating impact of LBT on wine quality and the economic losses suffered in 2001, many wineries have now initiated a zero

tolerance limit for ladybeetles in harvested grapes delivered to the wineries (Booram, 2006 unpublished). Although rejecting all grapes that contain Lady Beetles is one strategy to employ, if another MALB or 7-spot outbreak were to occur, rejecting all grapes with ladybugs may not be sustainable for the industry.

The study of pre-fermentation silicone treatments on juices to reduce MPs in finished wines without altering concentrations of other volatiles has only recently been investigated. In this study 5 mm rings of silicone were used, in a concentration of 40 g silicone / L of model juice, which was spiked with 50 pg/mL of MP (Ryona *et al.*, 2012). The result showed that a higher temperature of silicone contact had a better effect on the removal of both IPMP and IBMP (Ryona *et al.*, 2012). In addition, it was found that after two days of silicone contact at 25°C, an equilibrium had been reached between the silicone solid and the tainted model juice liquid phase, resulting in a higher than 95% removal; whereas it did not appear that equilibrium was ever reached within four days in tests at 16 °C (Ryona *et al.*, 2012).

Silicone treatment was also performed in actual juice in order to ensure that silicone could be used to reduce MP concentrations without affecting the other wine volatiles. The silicone was able to remove 53-93% of MPs in testing four different juices or musts with native or spiked IBMP or IPMP in a range of 2.7 to 61.2 pg/mL (Ryona *et al.*, 2012). In the test of 2007 Cabernet franc rose, the native 7.7 pg/ml of IBMP was removed below their limit of detection (1.2 pg/mL); however, the same level of removal was not achieved in testing 2008 Cabernet franc red juice with 7.4 pg/ml of IBMP with different contact dose and time. The use of silicone does appear to be a potential strategy for the removal of MP's from tainted juice without affecting most wine volatiles but this strategy is in its early development phase. The optimal contact dose and time for efficient removal of MPs from different types of juices and musts using

this strategy is required, followed by wine fermentation of the treated juices. This strategy partnered with other fining agents for MPs removal could be a potential strategy to remove MP's down below sensory threshold.

Molecularly imprinted polymers (MIP) are polymers that are able to bind to the target molecule, by having a cavity that is designed for that molecule (Kelm, 2012). This allows MIPs to be added to a solution containing a cocktail of molecules and the MIP will bind to the specific target molecule at a high percentage. However MIPs are also known to bind to a low percentage of non-target molecules. First used to decaffeinate coffee or tea, this idea spurred the idea that it could be used in wine to remove unwanted compounds (Kelm, 2012). When a methoxypyrazine specific MIP is added to methoxypyrazine tainted wine, a MIP-methoxypyrazine will form. These MIPs are found to be highly selective, sensitive and specific, and once bound to the target molecule, are removed by chromatography or solid phase extraction (Kelm, 2012). This method proved to be able to remove 25% to 99% of the IBMP found in the tainted wine, without compromising the property of the juice as noted in a patent application (Kelm, 2012).

A question of concern is which non-target compounds the MIP will bind and remove. These compounds being removed could affect the overall composition on the wine. In the study the MPs being removed are removed down past the sensory threshold, however what is the starting concentration of these compounds in the juice, and would they still be removed down past the sensory threshold had a higher starting concentration been used? Based on these questions it is not clear whether MIPs would be a desired method of MP removal, due to the possibility of affecting the flavor of the wine.

The approach from our lab was to develop a new protein-based fining strategy to provide winemakers with a tool to reduce the level of MPs in grape juice to below detection threshold using techniques and methods they were already familiar with. Protein based fining agents used in the industry, albumin and casein, were tested, but were found ineffective (Pickering *et al.*, 2006).

In developing a method to reduce the taint, if a protein was found that already had affinity to MPs, it could be exploited for its natural binding properties and developed into a fining agent for MP removal. Did such a family of proteins exist?

2.4 Odorant Binding Proteins, Pheromone Binding Proteins and the Lipocalin Family

Aromas move through our olfactory system, however how this mechanism occurs is still somewhat poorly understood (Hajjar *et al.*, 2006). Odorants enter the olfactory system by the thousands and are detected and discriminated by olfactory receptors (Tegoni *et al.*, 2000). Mammals and insects both have olfactory receptors which are protected from the air by a layer of aqueous mucus. The odorants and pheromones, which are volatile and hydrophobic in nature, require a delivery mechanism to cross this hydrophilic barrier to reach the receptor (Spinelli *et al.*, 1999). Carrier proteins that could transfer the hydrophobic odorants and pheromones across this hydrophilic barrier to their receptors were discovered and are known as Odorant Binding Proteins (OBPs) (Pelosi and Maida, 1995) and Pheromone Binding Proteins (PBPs) (Novotny, 2003), both of which are part of the lipocalin family (Flower, 1996).

Several biological functions in olfactory detection have been proposed for lipocalins based on their ability to capture volatile odorants in the early events of olfactory perception (Flower, 1996). In this respect, OBPs function as the first selective filter and may be good candidates for biotechnology interest to bind to and remove chemical compounds present in the air at traceable amounts, which cannot even be perceived physiologically (Flower, 1996).

Members of the lipocalin protein family are typically small secreted proteins which are characterized by a range of different molecular recognition properties; they have the ability to bind small, hydrophobic molecules (Flower, 1996). The lipocalins are a large and ever expanding group of proteins which are, however, very structurally and functionally diverse (Flower, 1996). The lipocalin family has been connected with the transport of mammalian pheromones due to easily observable protein-pheromone interactions (Flower, 1996). The lipocalin fold is a highly

symmetrical all β protein dominated by a single eight-stranded antiparallel β-sheet closed back on itself to form a continuously hydrogen bonded β-barrel, which, in cross section has a flattened or elliptical shape and encloses an internal ligand binding site (Flower, 1996). The eight β-strands or the barrel labelled A-H, are linked, these seven loops labelled L1-L7 are all typical short β-hairpins except loop L1, this is a large helical loop which forms a lid folded back to close the ligand binding site (figure 2.2) (Flower, 1996).

Figure 2.2 Schematic drawing of general Lipocalin: the 8 β-sheets connected β hairpins (modified from Flower, 1996)**.**

The molecular basis of both olfaction and taste is rather less well understood than the mechanisms underlying the other physical senses. Thus the discovery of specific proteins, usually referred to as OBPs, associated with olfactory tissue, which are able to bind odorant molecules with high affinity, have generated considerable interest (Flower, 1996).

Odorant-binding proteins (OBP) are low molecular weight, soluble proteins secreted by glands of the respiratory nasal mucosa that bind to hydrophobic odorants, and are thought to act as carrier proteins to deliver odorants to olfactory neurons (reviewed in Tegoni *et al.*, 2000). The first vertebrate OBP to be identified was from bovine nasal mucus, characterized by its ability to bind to pyrazines (Pelosi *et al.*, 1982). Mammalian OBP have now been identified in a variety of species including cow, pig, rabbit, mouse, rat, elephant and human (reviewed in Nespoulous *et al.*, 2004). Different subtypes of OBPs exist within the same animal species that bind to distinct classes of odorants with micromolar affinity (Briand *et al.*, 2000; Tegoni *et al.*, 2000).

It was the observation that members of the OBP family had high affinity to methoxypyrazines that led us to exploit this property to focus on this protein family to develop a fining agent to remove LBT from wine.

2.4.1 Rat OBP

Binding studies on the rat subtypes of OBPs revealed that rat OBP-1 had high affinity for aromatic heterocyclic odorants like pyrazines with a K_d value (how tightly the pyrazine binds to the Protein) for IBMP of 1.7 µM (Briand *et al.*, 2000). Rat OBP-2 was selective for aliphatic long chain aldehydes and carboxylic acids (Lobel *et al.*, 2001) whereas rat OBP-3 had ligand specificity that differed from that of OBP-1 and OBP–2, interacting strongly with odorants that had saturated or unsaturated ring structure (Lobel *et al.,* 2001). The rat OBP-1 cDNA was cloned, heterologously expressed in the yeast *Pichia pastoris* and secreted into the growth medium at 80 mg/L in a form indistinguishable from the native protein (Briand *et al.*, 2000). Based on the high affinity of OBP-1 to methoxypyrazines and a readily available source of OBP-

1 from the heterologous yeast expression system, the recombinant rat OBP-1F protein was a prime candidate protein for testing its ability to bind to MPs in a wine matrix. After gaining permission from Dr. Pernollet, the principal investigator that established the expression system in *Pichia* for OBP-1f, the Inglis lab purified the protein and tested its ability to bind to MPs in a juice/wine matrix. It was determined that due to the acidic environment of the juice/wine matrix, the protein, which is a dimer at neutral pH, was not able to bind to the methoxypyrazine most likely due to conversion to the monomeric form at the acidic pH (Boorom, 2006 unpublished). This result led to the search for other candidate proteins that bind MPs but can function at acidic pH (Table 1).

Table 2.1 Proteins that were considered as possible candidates to use in binding assays to bind MPs

Protein	*Kd*	Tertiary structure	Funtional pH
Rat OBP-1f	1.7µm(IBMP)	Dimer	7.5 (Briand *et al.*, 2000)
pOBP	0.8 µm (IBMP)	Monomer	3.5 (Burova *et al.*, 1999)
mMUP	1.8 µm (IPMP)	Monomer	5.5 (Lucke, 1999)
hOBP	0.9 µm (IBMP)	Monomer	7.5 (Briand, 2002)

2.4.2 Porcine OBP (pOBP)

Several OBPs have been isolated and extensively characterized among mammals, including porcine OBP, which is primarily purified from nasal tissues of castrated male pigs (Guiraudie-Capraz *et al.*, 2005). The biological function of OBPs in general, and pOBP in particular still are not fully known, they have been demonstrated to bind to a wide range of odorant ligands of different chemical structures including IBMP (Paolini *et al.*, 1999).

The pOBP is a functional homodimer at neutral pH, however it has been proven to still be a functional monomer at acidic pH (Paolini *et al.*, 1999). pOBP was found to have an affinity for methoxypyrazines greater than that found for bovine OBP, along with an affinity for a wide range of nutty and green smelling odorants (Herent *et al.*, 1995). pOBP is an example of a functional monomer active at an acidic pH of 3.5 for the binding of methoxypyrazines. The association for IBMP did not significantly change in the acid pH environment; it was 0.92 µM compared to 3.19 µM (Burova *et al*, 1999). No experimental research has been carried out to date on the binding affinity of pOBP with the methoxypyrazine of interest for ladybeetle taint, IPMP. An isoform of the pOBP, the piglet OBP (plOBP), is a protein that differs from the pOBP purified from adult pigs by an additional lysine residue at the C-terminal end (Guiraudie-Capraz *et al.*, 2005). It had been successfully expressed in a *P. pastoris* expression system and shown to functionally bind IBMP (Guiraudie-Capraz *et al.*, 2005).

2.4.3 Mouse MUP

Rodent urine contains an unusually large amount of protein, and this led to extensive studies in rats and mice leading to the identification of Pheromone Binding Proteins in urine (Flower, 1996). The major protein component in mouse urine is referred to as mouse major urinary protein, which is also found in the liver of the mouse (Cavaggioni and Mucignat-Caretta, 2000). It is able to bind to a number of different odorant molecules, which suggests that it is a transport protein. Studies done by Cavaggioni and Mucignat-Caretta in 2000, suggest that mMUP acts as a pheromone transporter, suggesting that this protein is a Pheromone Binding Protein. Odorant Binding Proteins have a homology with urinary proteins, which are a subfamily of proteins found in abundance in the urine of many animals. mMUPs provide a small range of identifying information about the donor animal, when detected by the vomeronasal organ of the receiving animal.

mMUP is a lipocalin that binds volatile pheromones and is part of the chemo-signalling system of mouse scent markers. mMUP is able to bind to a variety of ligands and it is thought that this unusual binding ability may be what allows the binding of primary aliphatic alcohols and of pheromone molecules such as HMH (6-hydroxy-6-methyl-3-heptonan), NPN (*N*-phenyl-naphthylamine), SBT (2-*sec*-butyl-4,5-dihydrothiazole) as well as IBMP and IPMP (Pertinhez e*t al.*, 2009) to the protein binding pocket. mMUP is a functional monomer at acidic pH, however experimental research on the binding affinity of mMUP with the methoxypyrazine of interest (IPMP) has only been done at a pH of 5.5 (Lucke, 1999).

2.5 Protein Expression Systems

In order to exploit these proteins which possess natural affinity to MPs, it was important to establish a cost effective expression system to produce large amounts of the proteins for binding studies to avoid isolating the proteins from their native source in pig and mouse. This led to the following questions, what expression system should be used and how would the recombinant protein be purified and subsequently used in an MP removal system.

In investigating expression systems it is necessary to choose a system that is able to synthesize large quantities of the protein, while still ensuring that the protein is folded properly, can be easily purified and is found to still be biologically active. Possible expression systems are E.*coli*, yeast, and virus infected insect cells, all of which offer advantages and disadvantages in recombinant protein expression (Schmidt and Hoffman, 2002).

The tertiary structure of OBPs and MUPs is an eight-stranded, antiparallel, symmetrical β-barrel fold. This structure is important as the active site for ligand binding occurs within the barrel of the protein (Schmidt and Hoffman, 2002). Due to this tertiary structure, it is important to choose the appropriate expression system to synthesize this protein, in order to ensure that the protein is folded properly and still biologically active. *E. coli* is the oldest and most commonly utilized system of production for recombinant proteins, as the bacterial expression system is easy to handle, cost-effective, and can produce a high yield (Schmidt and Hoffman, 2002). The down side of this type of expression system is that often overproduction of the proteins occurs, which leads to mis-folding and segregation into insoluble aggregates known as inclusion bodies, resulting in the production of biologically inactive proteins (Schmidt and Hoffman, 2002). *S. cerevisiae* is a eukaryotic expression system which is also an easy system to handle. The

advantage of this system is its ability to perform post translational modifications, although it has been known to hyperglycosylate proteins, and this is why researchers usually tend to prefer *P. pastoris* (Schmidt and Hoffman, 2002).

In reviewing the literature for the two target proteins of interest for this thesis, plOBP and mMUP, both proteins had been successfully expressed in *P. pastoris* and purified from the growth media for further study (Guiraudie-Caprez *et al.*, 2005; Lucke, 1999). Therefore, the *P. pastoris* expression system was chosen to clone and express these proteins.

2.5.1 *Pichia pastoris* Expression System

Using *P. pastoris* as an expression system was developed over 20 years ago in order to obtain high level of recombinant protein expression (Invitrogen). A eukaryotic expression system offers the increased ability in expression systems to process proteins, at an easier and faster level, while remaining inexpensive (Invitrogen).

P. pastoris, can produce recombinant proteins both intracellular and extracellular, while being able to also perform the necessary eukaryotic post-translational modifications (Cereghino and Craig, 2000)

Secretion is the best mode of protein production since the organism secretes only very low levels of its own proteins, which facilitates protein purification from the growth media. The secreted protein requires the presence of a signal sequence to target it into the secretory system, such as the *S. cerevisiae* α- mating factor sequence. The basis behind induced protein expression in the *P. pastoris* expression system stems from the fact that some of the enzymes that are

required for methanol metabolism are only present when cells are grown in methanol (Cereghino and Craig, 2000).

Expression of any foreign gene in *P. pastoris* requires the insertion of the gene into an expression vector, the linearization of the expression vector followed by its introduction into *P. pastoris* by electroporation, insertion of the expression cassette into the genome of *P. pastoris* by homologous recombination and finally the examination of potential expression strains for the foreign gene product. It is important when expressing heterologous proteins that the protein of interest is able to be isolated from other native secreted proteins (Humes, 2007 unpublished).

2.5.2. Codon Bias for Efficient Expression

Final protein yield can be significantly enhanced by manipulation of the factors that influence gene expression and product stability (Sreekrishna *et al.*, 1997). A neutral theory of molecular evolution suggests that mutations at degenerate coding positions should be selectively neutral and, thus synonymous codon choice random (Sreekrishna *et al.*, 1997). All organisms investigated to date show some general bias towards a subset of the 61 possible sense codons (Sinclair and Choy, 2002). This so called "major codon preference" model of codon bias stems from the observation that preferred codons are consistent across the genes within a given genome and correlate with the most abundant aminoacyl-tRNAs present in the cell (Sinclair and Choy, 2002). In order to maximize protein translation rate, coding sequences should use codons matching the most common tRNAs (Sreekrishna *et al.*, 1997).

Since the codon preference of *S. cerevisiae* has been well characterized, the increasing interest in *P. pastoris* as an expression host led to a thorough analysis of synonymous codon usage in the methylotrophic yeast (Sinclair and Choy, 2002). The influence of codon usage on

the expression level of individual heterologous proteins in yeast has been investigated and there seem to be parameters that are influential on the expression level. The one main parameter that has been shown to have a statistically significant association with the expression level is that less AT rich regions will result in a greater expression level (Boettner, 2007). The complete optimization of coding regions towards the codon bias of the host cell has led to an average 10-to-50 fold increase in heterologous protein production in a variety of host cells expressing an equally diverse range of foreign proteins (Sinclair and Choy, 2002).

2.5.3 Targeting Heterologous Proteins in *P. pastoris* into the Yeast Secretory System and Processing the Preprotein into the Mature Form

P. pastoris is a methylotrophic yeast that is not known to secrete many proteins, as most proteins produced remain intracellularly (Invitrogen). For this reason, if a yeast secretory sequence at the N-terminal of the heterologously expressed protein is added, the protein of interest will be secreted through the yeast secretory system and can more easily be purified from the growth media (Invitrogen). This can be accomplished by cloning the coding region of the gene of interest in frame with the *S. cerevisiae* α-mating factor preprosequence in the expression vector used in the *P. pastoris* system (Invitrogen).

In addition to having a protein secretory signal fused to the heterologously expressed protein, this signal sequence must be cleaved off to release the mature protein of interest into the growth media. Therefore, between the α-mating factor preprosequence and the mature protein sequence of the heterologous protein, there must be a Kex2 cleavage site to allow the secretory signal to be cleaved off from the protein of interest as it moves through the yeast secretory pathway (Invitrogen).

2.5.4 Sequencing and Cloning plOBP and mMUP into PIC9 Expression Vector

The complete amino acid sequence is known for a number of vertebrate OBPs, and from these, a large cDNA library has been constructed, which include the cDNA of plOBPs (Scaloni *et al.*, 2001) and mMUP (Flower, 1996). The mature sequence for plOBP was determined to be 158 amino acids long (Scaloni *et al.*, 2001) and that of mMUP was 152 long (Flower, 1996). Due to the relatively low cost of synthesizing DNA sequences today from a published sequence versus the costs to clone the gene from an original animal source, both plOBP and mMUP codon optimzied nucleotide sequences were synthesized and cloned into PIC9**.** Once the two sequences were obtained, they were inserted individually into the pUC57 cloning vector prior to being subcloned into the PIC9 expression vector.
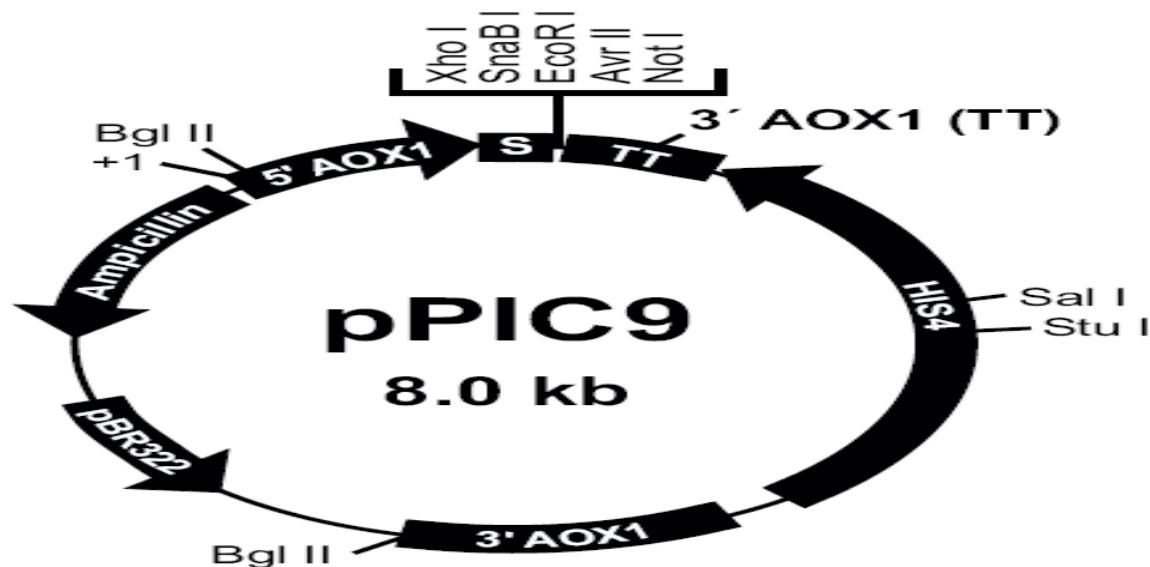
Figure 2.3. The schematic of the Expression vector, with designed nucleotide insert (modified from Invitrogen *Pichia pastoris* manual).

2.6 MP Removal System and MPs Measurement

The goal of this project was to develop an MP removal system that would function in grape juice and/or wine to reduce MPs to acceptable detection levels. Therefore in developing this system, not only was a technique required to pull the protein-MP complex out of solution, but also a sensitive measurement method for measuring low concentrations of MPs was required.

2.6.1 Bentonite Fining

It was important to look at options for capturing the soluble protein-MP complex so the complex could be removed from solution prior to measuring the residual MPs left in solution. Options to consider include: covalently binding the protein to an inert support and passing the solution containing MPs over the support material; filtering the complex through a membrane with a small enough pore size that the protein-MP complex will not flow through; or capturing the protein-MP complex with an insoluble counterfining agent such as bentonite.

Bentonite is a type of clay that was first discovered in the 19th century, it is distinct from other clays in that bentonite is formed from volcanic ash. It has super water absorption characteristics and some even claim medicinal properties when the clay is consumed or used externally. Bentonite is insoluble and negatively charged and therefore acts by electrostatic interactions attracting positively charged compounds (Blade and Boulton, 1988). Since proteins tend to be below their PI at the pH of juice and wine, and hence positively charged, bentonite was a candidate material to investigate for capturing the protein-MP complex from solution (Blade and Boulton, 1988).

The effectiveness of bentonite depends upon the method of preparation and addition, the quantity employed, the temperature and the pH. Bentonite is a complex hydrated aluminum silicate with exchangeable cationic components: (Al, Fe, Mg) Si_4O_{10} $(OH)_2$ (Na^+, Ca^{++}). The

most commonly used form in North America is sodium bentonite (Blade and Boulton, 1988). The mechanisms of protein removal are adsorptive interactions between positively charged proteins and negatively charged bentonite.

At a certain pH, the positive and negative charges of each protein fraction are equal and the protein is least soluble. This pH value is known as the isoelectic point, or isoionic point (PI), of the protein. The lower the difference between the juice or wine pH and the PI of the protein fraction, the lower is the net charge on that protein fraction. Therefore, the isoelectic properties of proteins influence not only their natural tendency to precipitate but also their affinity to be removed with various agents. The PI of mMUP and plOBP is around 4.7 and 4.2 respectively, and this is why they are able to be removed at juice pH of pH 3-4, as they will have a positive charge.

2.6.2 Quantification of MPs Using GC/MS

To date there has not been a system developed that could quantify the amount of MPs in a solution without many areas of speculation and uncertainties. Trace volatiles can be extremely difficult to detect and requires a high level of instrumental sensitivity. First identified in Cabernet Sauvignon grapes in 1975, IBMP was tentatively linked to the vegetative character of Cabernet Sauvignon wines, however this first effort was qualitative and unable to describe the sensory impact of IBMP (Blake *et al.*, 2009). Gas Chromatography / mass spectrometry (GC/MS) technology and the use of liquid-liquid extraction with a deuterium labeled analog of IBMP has allowed for the isolation and quantification of MPs in wine close to their detection threshold (Blake *et al.*, 2009). In general, techniques involving liquid-liquid extractions are overly laborious and time consuming. A recent method with extremely high sensitivity, accuracy and precision has been developed and validated that involves the optimization and quantification of

all MPs by the use of headspace (HS) solid phase microextraction (SPME) coupled with GC/MS and deuterium labeled internal standards for each MP compound. This method allowed quantification of MPs in juice and wine down to ultra-trace concentrations (Kosteridis *et al*., 2008).

3. Methods and Materials

The cDNA sequence encoding mature piglet OBP (AF436848) and the mouse MUP2 (AJ309921) were designed for heterologous expression in *P. pastoris* as previously described in our PCT patent (Inglis *et al.*, 2010 and included in appendix 1).

3.1 Expression of Recombinant Proteins (plOBP and mMUP) from *P. pastoris*

P. pastoris with recombinant proteins (plOBP or mMUP) were grown in Buffered Glycerol-Complex Medium (BMGY) for a quick biomass build-up, and then transferred to Buffered Methanol Medium (BMM) for inducing protein expression over an induction period of two days.

Yeast colonies of *P. pastoris* were firstly inoculated into 1 L of BMGY (1% yeast extract, 2% peptone, 0.1 M potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1% glycerol) from YPD plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar). Inoculated 1 L BMGY medium was mixed well, aliquoted into 10 baffled flaskes (100 ml in each) and then incubated overnight in a temperature controlled shaker at 29°C, 210 rpm. The overnight cultures were grown to log phase, to an OD600 between 2 and 6. Cultures were then spun down at 2000g, 4°C for 10 minutes, supernatant was discarded, and yeast pellets were re-suspended in 200 ml of BMM (0.1 M potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1%

methanol). BMM with re-suspended *P. pastoris* was then aliquoted into two baffled flasks (100 ml in each) and incubated again at 29°C with shaking at 210 rpm for protein induction. Induction was carried out for two days and 1 ml of sterile filtered 100% methanol was added daily to a final concentration of 1% (v/v) to maintain induction. Each day a 1mL sample was removed for observation and for analysis.

3.2 Purification of Recombinant Proteins

The 2-day BMM culture containing recombinant plOBP or mMUP was collected and centrifuged at 3000 g for 10 min at 4°C. The supernatant was collected, filtered through a 0.45 μM membrane (Millipore, Billerica, MA) and pooled. Filtered supernatant was divided into four aliquots (50ml each) and each aliquot was placed in a 10kDa molecular-weight-cut-off (MWCO) dialysis tubing (spectra por), and dialyzed against 20mM Tris-Cl solution pH 8.0 in a 4 L beaker for four days at 8°C to reach an equilibrium. Dialyzed supernatant was then purified in a single anion exchange chromatography step on an Akta Explorer Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare, Piscataway, NJ), using a Q ceramic HyperD F strong anion exchanger. Equilibrated supernatant was loaded onto a Hi Trap Q sepharose 1mL column which was already pre equilibrated with 20mM Tris-Cl, pH 8.0. Pre-equilibration ensured that both supernatant and column were at the same pH 8.0. Sample was loaded using a 50 ml superloop (GE Healthcare, Piscataway, NJ). Tris-Cl buffer (20mM, pH 8.0) was used as equilibrium buffer and elution was carried out with a linear gradient of 0 to 1.0 M NaCl in 20mM Tris-Cl, pH 8.0. Fractions (0.5 mL) were collected and analyzed by SDS PAGE, and those with protein of interest in high purity were pooled together. Purified fractions were dialyzed against MQ water for 2 days at 8°C for de-salting, with MQ water changed twice.

Desalted protein was collected and 6.5 mL from each 50 mL purification was then aliquoted into one 15 mL falcon tubes and frozen at -80°C for two hours. Frozen protein was then lyophilized for approximately 2 days.

3.3 Determination of Recombinant Proteins

3.3.1 Visualization of mMUP and plOBP by SDS PAGE

The expression and purity of protein of interest in yeast cultures was visualized by sodium dodecyl sulfate polyacrylamide gel electropohoresis (SDS PAGE) with a 15% resolving gel (Table 3.1) and a 5% stacking gel (Table 3.2) using the mini-Protean®3 Cell system from BIORAD. Resolving gel was allowed to set for 30 minutes before samples were loaded.

Table 3.1 Components of 15% resolving gel for SDS PAGE

Volume	5 ml
MilliQ H_2O	1.1 ml
30% Acrylamide	2.5 ml
1.5 M Tris (pH 8.8)	1.3 ml
10% (w/v) SDS	50 μl
10% (w/v) APS	50 μl
TEMED	5 μl

Table 3.2 Components of 5% stacking gel for SDS PAGE

Volume	5 ml
MilliQ H_2O	3.4 ml
30% Acrylamide	830 µl
1.0 M Tris (pH 6.8)	630 µl
10% (w/v) SDS	50 µl
10% (w/v) APS	50 µl
TEMED	5 µl

Protein samples (20 µL) were mixed with 5µL of 5×sample buffer (0.05 M Tris pH6.8, 16% glycerol, 1.6% SDS, 0.02% mercaptoethanol, 0.08% w/v bromophenol blue, stored at - 30°C), mixed well, and heated at 95°C for 6 minutes. Samples were then quickly transferred on ice, spun briefly, and then loaded into the wells of the gel. One well was reserved for 10µL of Precision Plus Protein[TM] Unstained Standards (BIORAD, USA), used as a size reference.

The gel electrophoresis was carried out with a PS500XT DC power supply (Hoefer Scientific Instruments, San Francisco) at 100 volts for 1.5 hours. It was then removed from the electrophoresis tub and fixed in a fixing solution (40% ethanol, 10% glacial acetic acid) for one hour, followed by staining with Coomassie Gel Staining Solution (0.1% w/v Coomassie R-250, 25% methanol, 10% glacial acetic acid) overnight. On the following day, the gel was destained in gel destaining solution (100% methanol, 10% glacial acetic acid), and then visualized with Gel-Doc 1000 and Multi-Analyst v1.1 (BIO-RAD Inc., USA).

3.3.2 Quantification of Protein by Bradford Assay

Protein concentration of both expressed and purified proteins were determined by Bradford using Bovine Serum Albumin (BSA) as a standard protein. BSA stock solution (2 mg/mL, Pierce, USA) was diluted to a serial of concentrations, 0, 100, 250, 500, 750, 1000 µg/mL, and tested along with protein samples to build up a standard curve for reference.

In order to set up a Bradford assay, 100 µL of samples were added to 3 mL of Bradford Reagent (Sigma-Aldrich, USA) in a 3 mL plastic Cuvette, same as prepared BSA standards at different concentration. All cuvettes were mixed by inversion and then incubated for 5 minutes. Absorbance readings were then taken at a wavelength of 595 nm by the Spectronic Genesys 2. Protein concentration in the samples were then determined from the standard curve by interpolation.

3.4 Protein – MP Interaction and Binding Reactions

3.4.1 Preparation of Juice

The frozen juice, both Riesling and Chardonnay donated from Niagara Vintage Harvesters, Inc., Virgil, ON, was thawed in the 4°C cold room for three days. In order to remove debris and indigenous microorganisms, the juice was filtered through coarse (8 µm), medium (2-8 µM) and fine pore-size (0.5-2 µM) Bueno Vino Mini Jet pad filters.

A sample of the juice was taken and the titratable acidity (TA) and pH were measured as outlined in Zoecklein *et al*., 1995, and an aliquot of juice was de-acidified by the use of calcium carbonate to attain the desired pH (Zoecklein *et al.*, 1995). The de-acidified juice was added to the filtered juice in order to obtain a pH value of 3.5 for the total juice matrix. The juice was

then sterile filtered, through a Millipore cartridge Durapore optiseal unit. After sterile filtration, the juice was aliquoted into 50mL samples and frozen.

3.4.2 Binding Reactions

The freeze dried preparations of plOBP and mMUP were resuspended in 1.64 mL phosphate citrate buffers at pH 3.0, 3.5, and 4.0 as well as in Chardonnay juice and Riesling juice pH 3.5, to a final concentration between 40-50 µM and incubated for 30 minutes. The protein solutions, in a 2 mL volume, were spiked with IBMP or IPMP at 300 ng/L and allowed to incubate in a glass vial for 2 hours at room temperature on a gel shaker. 3 g/L bentonite was added to vials and incubated for 1 hour, in order to remove protein and protein-ligand complex. Vials were then centrifuged at 2500g for 55 minutes and filtered through a 0.22 µM Durapore filter unit (Millipore) in order to remove bentonite–protein complex as well as any residual bentonite from the reaction. The concentration of the residual MP fraction was then determined using HS-SPME GC/MS. The limit of quantification for the compound IBMP was determined to be 6 ng/L and for IPMP was 2 ng/L, determined from10-times of signal/noise. The limit of detection for MPs in the present system for IBMP was 1.8 ng/L and for IPMP was 0.6 ng/L based on three times the signal noise. All data has been reported to the limit of quantification in figures of this thesis.

With the bentonite filtration system, the starting concentration of MPs in the reaction was tested in order to confirm the spiked starting concentration. The residual MPs after filtering through 0.22 µM Durapore filter were tested to determine whether filtration alone would affect MP concentration. The residual MPs after bentonite addition and filtration through the 0.22 µM filter were tested to determine whether they have an effect on MPs removal. The residual MPs

after the protein binding reaction, bentonite fining and filtration were tested to determine if proteins could further remove MPs from solution.

3.4.3 Quantification of MPs by GC/MS

Samples were prepared with a mixture of isotopically-labelled internal standards (d-IBMP and d-IPMP) that had been manufactured by Dr Ian Brindle and his lab. These standards were then diluted from their starting concentration of 1g/L to a final concentration of 300 ng/L of each internal standard, basified with NaOH, to increase the pH to approximately 6.6. 2 mL portions of the solution was prepared in the head space vials which contained approximately 30% w/v sodium chloride, in order to improve phase transfer, with a small stir bar, and were sealed with a cap for preservation. The sample was then extracted for 30 mins with stirring (1100 rpm) at 40°C by a HS-SPME fiber (Stableflex Divinylbenzene/Carboxen/PDMS; Supelco, Oakville, Ontario) inserted through the septum into the head space of the vial. After extraction, the fiber was carefully retracted and inserted into the GC/MS (Agilent 6890GC/5975B with an HP-5MS 5% Phenyl Methyl Siloxane column (30m, 0.25mm i.d., 0.25 11m film thickness), Agilent, Oakville, Ontario) inlet for sample desorption and analysis. The GC/MS program was as follows: in splitless mode, the injector heldup with no purge at 250°C for 5 mins for sample desorption then purged at 50 mL/min for 5 mins to clean the fiber. The oven remained at 40°C for 5 mins, ramped at 3°C/min up to 110°C, held for 1 min, and ramped at 25°C/min up to 230°C. Helium was used as the carrier gas at constant pressure (10.36 psi) with a nominal initial flow (1.2 mL/min). The MSD interface was held at 250°C while the temperature of the ion source was 230°C.

Identification was achieved using select ion monitoring. For IPMP and d-IPMP, respectively, selected mass channels were m/z=137 and 140, where these ions were used for

quantification. For IBMP and d-IBMP, selected mass channels were m/z=124 and 127, where these ions were used for quantification. All samples were analyzed to at least an n=6. Area ratios (area of a MP peak/area of corresponding d-MP) were calculated from chromatograms and correlated to concentration based on a standard curve.

4. Results

4.1 Expression of Protein in *Pichia pastoris*

The *P. pastoris* was grown for 16 -18 hours in Buffered Glycerol-Complex Medium in order to increase cell mass, as the presence of glycerol does not allow transcription to occur, therefore, the cell mass is able to increase, while no protein is being produced. The increased cell mass after being spun down was then collected and re-suspended in a buffered methanol media, as the gene was controlled by an alcohol dehydrogenase promoter, thus the presence of methanol as the single carbon source allowed for transcription to occur, and thus the increased cell mass was able to produce proteins. Expression of proteins occurred for two days (day 0 induced and grown two days) with the recombinant protein being excreted into this methanol-based induction media. Cultures were then spun down, yeast pellets were discarded, and the supernatant was kept for dialysis.

No protein was expressed in either the native *P. pastoris* harbouring the parent vector pPIC 9 or expression day 0 as evident in Figure 4.1. The band of protein sample being expressed over a two-day induction was apparent at ~18kDa (Fig. 4.1), as the molecular mass of the recombinant proteins have been reported to be 18.2 kDa for the plOBP and 18.7 kDa for the mMUP.
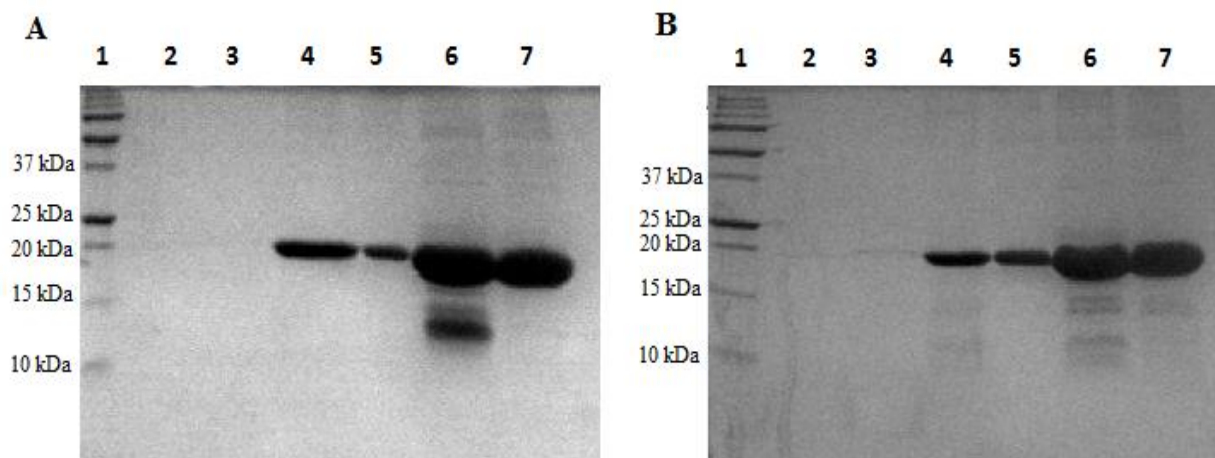
Figure 4.1 SDS PAGE gels of plOBP (A) and mMUP (B) from *P. pastoris* expression system. Lane 1 represents molecular weight markers (Precision plus Standard from Biorad), lane 2 represents expression samples from *P. pastoris* transformed with native PIC 9, lane 3 represents protein samples from expression Day 0, lane 4 represents protein samples after two-day expression, lane 5 represents protein samples after four-day dialysis, lane 6 represents purified protein samples from anion exchange chromatography, and lane 7 represents protein samples after rehydration of purified proteins.

4.2 Purification of Protein

Once identification of the protein was confirmed, the cell mass was removed and the 200 mL of supernatant was dialyzed for four days, in order to remove any smaller peptides and compounds that were expressed with the recombinant protein. The dialysis also allowed the proteins to be equilibrated to a pH of 8.0 in 20mM Tris Cl buffer for use in the Akta explorer (FPLC). 50 mL of the dialyzed media were run through a 1ml Q ceramic HyperD F column for each purification batch, and purified protein aliquots were obtained against a high salt concentration elution buffer (20mM Tris-Cl 1M NaCl). Fig. 4.2 shows a chromatogram produced by the FPLC, based on the monitor of absorbance at 280 μm. The increase in absorbance indicates the presence of protein and it can be observed that the purified protein was eluted as the salt concentration increased, from approximately 30% of 1M NaCl and continues until it was slightly higher than 70 %.

Protein purification was performed in order to remove any degraded proteins and native *Pichia pastoris* proteins that accumulated during the induction phase (Figure 4.1 A and B) as well as to concentrate the protein. The final volume of pooled fractions of purified protein obtained from the initial 200 mL of induction media supernatant was approximately 26 mL. The % yield for protein recovery was on average 78% for pIOBP and 62% for mMUP (Table 4.1). Final purified protein attained per L of induction medium was 19 mg/L for mMUP and 87 mg/L for pIOBP (Table 4.1 and Figure 4.1A and B).
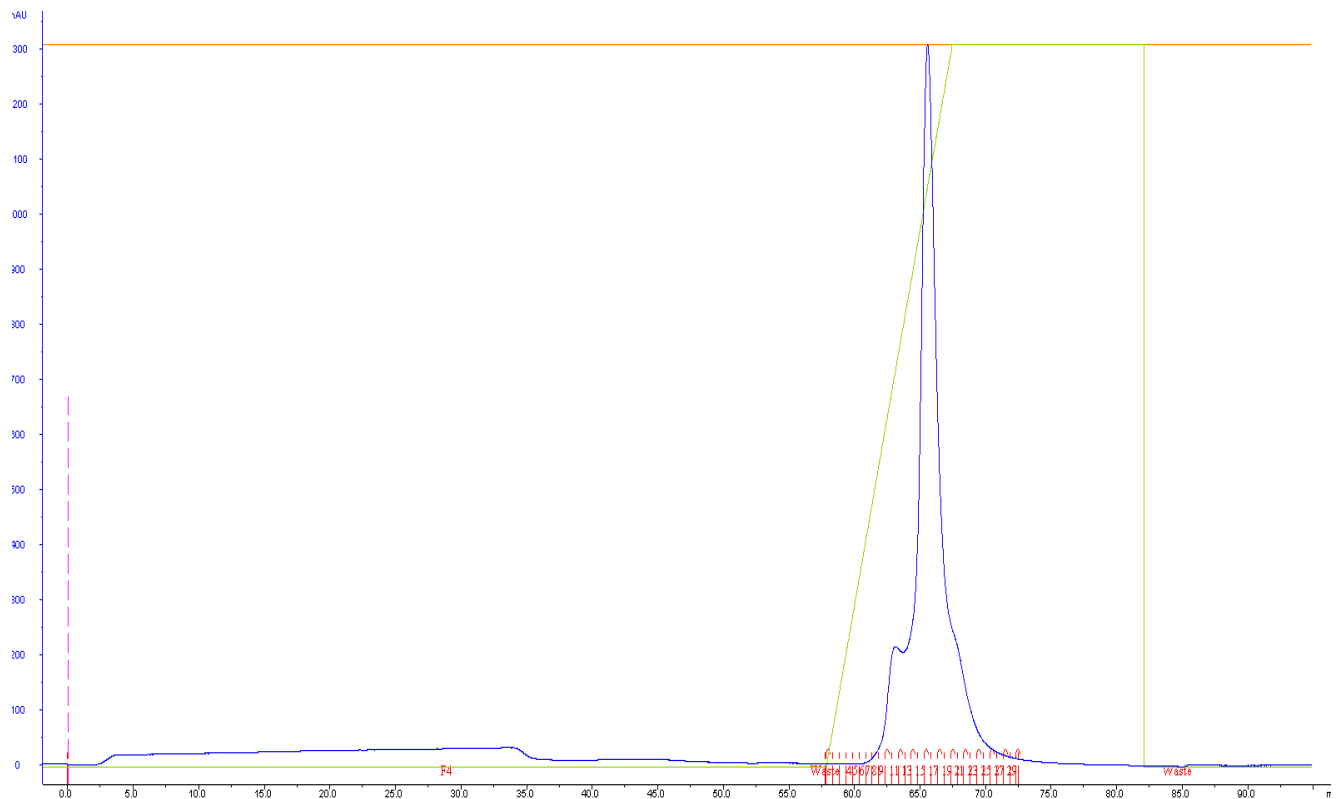
Figure 4.2 FPLC chromatogram of 50ml dialyzed supernatant from a two day expression of mMUP.

Table 4.1 Percent yield of purified protein after purification and dialysis.

Protein	Protein Expression at Day 2 of induction	Day 2 protein yield from 50 mL of induction medium	Purified Protein from 50 mL	% yield	Purified Protein per Litre of Induction medium
plOBP	111 mg/L	5572 µg	4347 µg	78%	87 mg/L
mMUP	31 mg/L	1538 µg	953 µg	62%	19 mg/L

4.3 Development of the Protein Removal System

4.3.1 Determination of the Optimal Amount of Bentonite for the Protein Removal System

The use of bentonite to remove proteins from solution is common practice in general wine making procedures, therefore it was necessary to determine how much bentonite would be needed in order to remove the recombinant proteins from solution, ranging in pH from 3.0 to 5.0. plOBP was used for the initial trials (Figure 4.3) followed by mMUP (Figure 4.4).
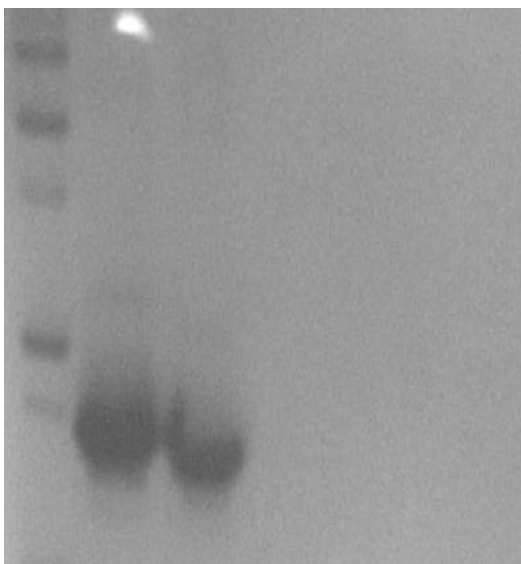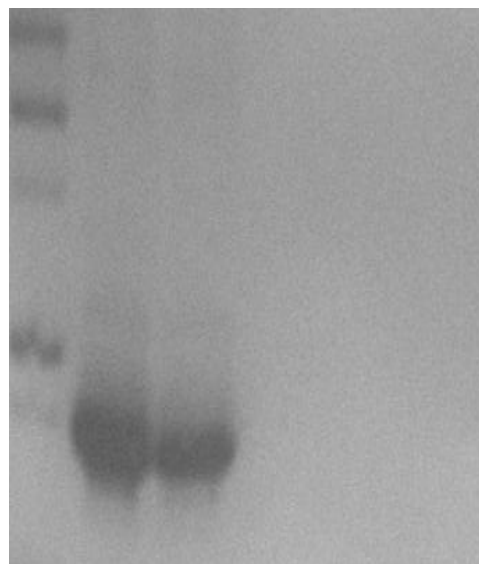
A
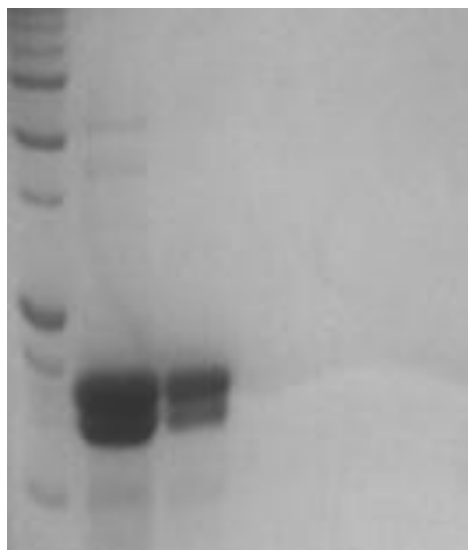
B
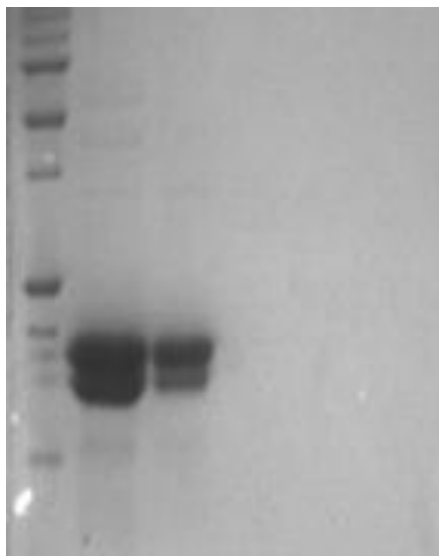
C
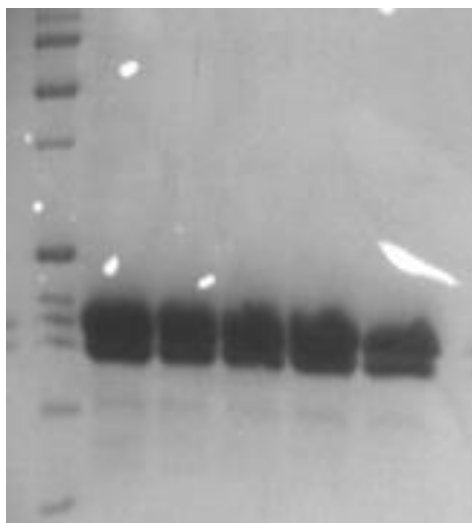
Figure 4.3. Bentonite fining trials at pH 3.0 (A), pH 4.0 (B) and pH 5.0 (C) with plOBP. All trials were done using a bentonite slurry in a 2 mL phosphate citrate buffer containing approximately 900-1100 µg/mL of plOBP. Lane 1 is the Marker, lane 2 is trials with 0 g/L bentonite followed by filtration, lane 3 is 1 g/L, lane 4 is 3 g/L, lane 5 is 5 g/L, and lane 6 is 7 g/L.

It is apparent that pH 5.0 is not an ideal pH to use when using bentonite to remove plOBP as not even 7g/L was able to remove the protein (Fig. 4.3); this is because the pH is above the isoelectric point of the plOBP which is 4.2. When this occurs then the protein is no longer positively charged and the bentonite is not able to bind to the protein thus not being able to remove it. However at both pH 4.0 and pH 3.0 sufficient protein removal is apparent when using bentonite concentrations of 3 – 7 g/L removing all of the protein (900-1100 µg/mL) and resulting in Bradford results of 0 µg/mL (Table 4.2).

Table 4.2 Bentonite fining trials using various concentrations of bentonite at pH 3.0, 4.0, and 5.0 with plOBP

	Protein Concentration after Bentonite fining		
References	**pH 3.0**	**pH 4.0**	**pH 5.0**
Control (starting [protein])	900 µg/mL	1034 µg/ mL	1094 µg/ mL
1g/L	250 µg/mL	379 µg/ mL	830 µg/mL
3g/L	0 µg/mL	0 µg/ mL	797 µg/ mL
5g/L	0 µg/ mL	0 µg/ mL	784 µg/mL
7g/L	0 µg/ mL	0 µg/mL	739 µg/ mL

A   **B**

Figure 4.4. Bentonite fining trials at pH 3.0 (A) and pH 4.0 (B) for mMUP. All trials were done using a bentonite slurry in a 2 mL phosphate citrate buffer containing approximately 850-950 µg/mL of mMUP. Lane 1 is the Marker, lane 2 is trials with 0 g/L bentonite followed by filtration, lane 3 is 1 g/L, lane 4 is 3 g/L, lane 5 is 5 g/L, and lane 6 is 7 g/L

When doing the same bentonite experiments with mMUP, only pH 3.0 and 4.0 were tested, as the isoelectric point of mMUP is 4.7, and is again below pH 5.0 therefore it is assumed that the bentonite would not function at that pH. At both pH 4.0 and pH 3.0 sufficient protein removal is apparent when using bentonite concentrations of 3 – 7 g/L removing all of the protein (900 µg/mL) and resulting in Bradford results of 0 µg/mL (Table 4.3).   For both plOBP and mMUP, bentonite concentrations of 3g/L were used in future MP-removal assays as this concentration was sufficient to remove all protein and is at the upper concentration that is used commercially (Blade and Boulton, 1988). If too high a concentration of bentonite is used, there is a greater chance of changing the juice/wine chemistry as bentonite will remove amino acids and can lead to altered taste and aroma (Blade and Boulton, 1988).

Table 4.3 Bentonite fining trials using various concentrations of bentonite at pH 3.0 and 4.0 with mMUP.

|	[Protein] after fining	
References	**pH 3.0**	**pH 4.0**
Control (starting [protein])	850 µg/mL	950 µg/ mL
1g/L	327 µg/mL	384 µg/ mL
3g/L	0 µg/mL	0 µg/ mL
5g/L	0 µg/ mL	0 µg/ mL
7g/L	0 µg/ mL	0 µg/mL

4.3.2 Determination of the Optimal Amount of Protein for the Protein Removal System

Protein binding curves were constructed in order to determine what the optimal concentration of protein required to remove the MPs. Since it was shown in earlier studies (Inglis *et al*., 2010) that pIOBP had a greater affinity for IBMP, IBMP was used in this initial study.

Binding reactions took place in pH 4.0 phosphate citrate buffer containing 300 ng/L of IBMP. Different concentrations of protein, from 3 µM to 60 µM were tested. Each reaction underwent bentonite fining followed by filtration to remove the protein-MP complex. In Fig. 4.5A the amount of residual IBMP after treatment with 0, 3, 15, 30, 45 and 60 µM of plOBP was plotted. It is apparent that as the concentration of protein increased, less IBMP was left in the buffer. This was further emphasized in Fig. 4.5B where the amount of IBMP removed was plotted against the same protein concentrations. Complete saturation of plOPB at the

concentration of IBMP tested was not obtained (Figure 4.5B). There was still residual IBMP left in the binding reaction after the 60 µM protein addition (Figure 4.5A). When referring to Fig.4.6, the gel indicates that the bentonite was not able to successfully remove all of the plOBP at 45 µM and 60 µM.

A
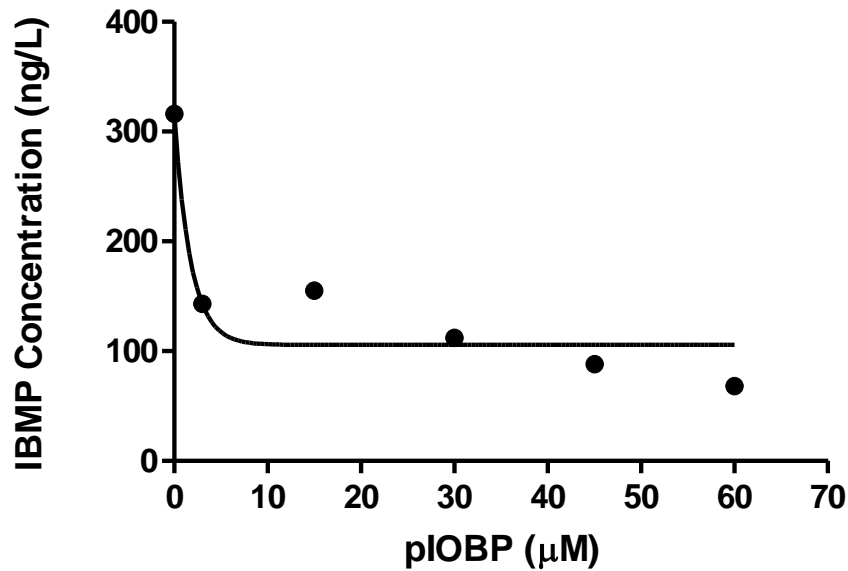
B
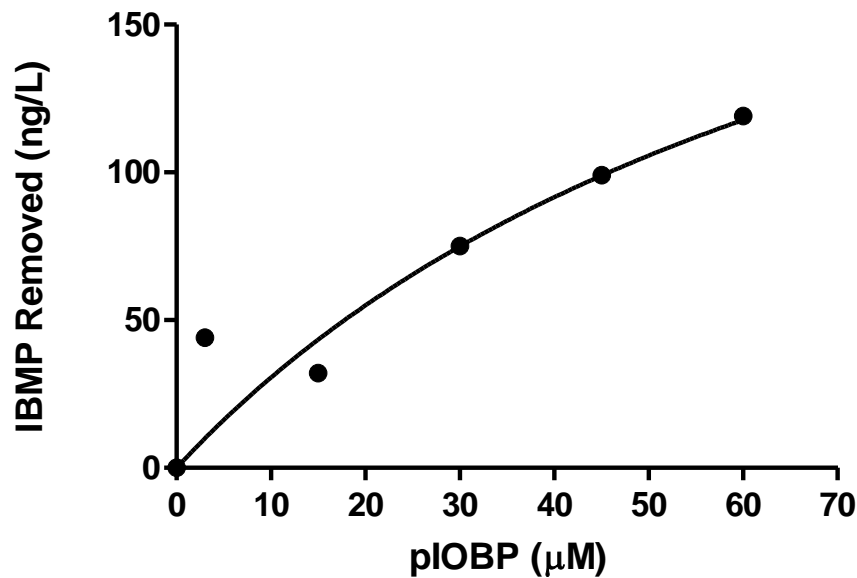
Figure 4.5 Binding of IBMP at 300 ng/L with increasing pIOBP concentrations. (A) The residual IBMP concentration was measured by GC/MS following bentonite fining and filtration. (B) Removed IBMP concentration was determined from the difference between the residual IBMP after bentonite fining followed by filtration with no protein addition and the residual IBMP after protein addition and removal by bentonite fining and filtration.
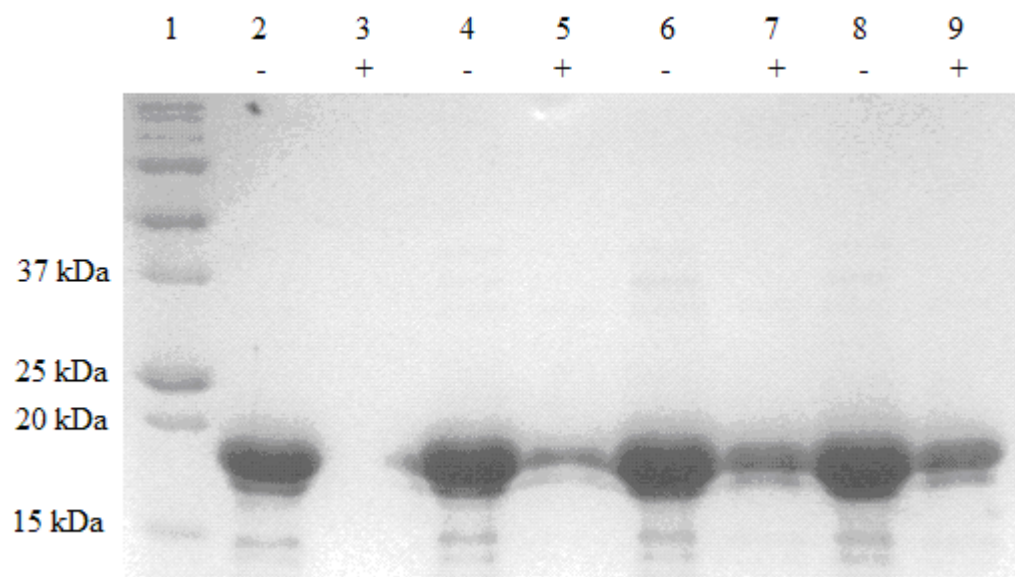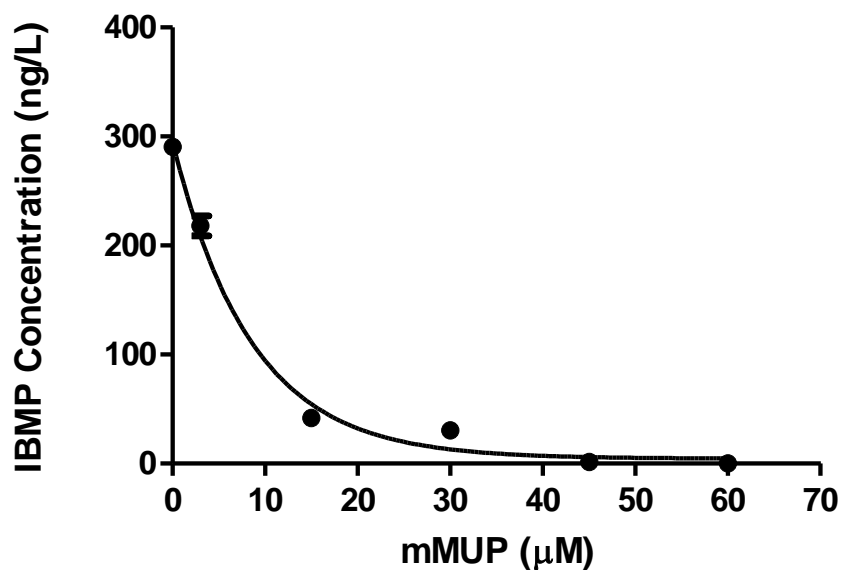
Figure 4.6 SDS PAGE gel of plOBP protein remaining after bentonite fining (3 g/L) and filtration of increasing amounts of protein. Lane 1 represents the Bio Rad marker, lane 2 is the 30 µM of plOBP with no bentonite fining. Lane 3 represents 30 µM plOBP after bentonite fining. Lane 4 represents 45 µM plOBP with no bentonite fining. Lane 5 represents 45 µM plOBP after bentonite fining. Lane 6 and 8 represent 60 µM plOBP with no bentonite fining. Lane 7 and 9 represent 60 µM plOBP with bentonite fining. Protein concentration increases from 30 to 60 µM, and the "-" indicates no bentonite, while the "+" indicates 3 g/L bentonite used.

In the binding reactions using mMUP, the protein was able to remove all 300 ng/L of IBMP over the protein concentration range tested. As seen in both Fig. 4.7 A and 4.7 B there was no remaining IBMP after an addition of mMUP at 60 µM. As well very little, only 7 ng/L of IBMP was remaining at 45 µM mMUP (Fig. 4.7A). From the gel it is apparent that although saturation may have been reached, it was again not possible for the bentonite to remove all of the protein at the 60 µM concentration and there was a faint mMUP band still apparent at 45 µM (Fig. 4.8).

A

B
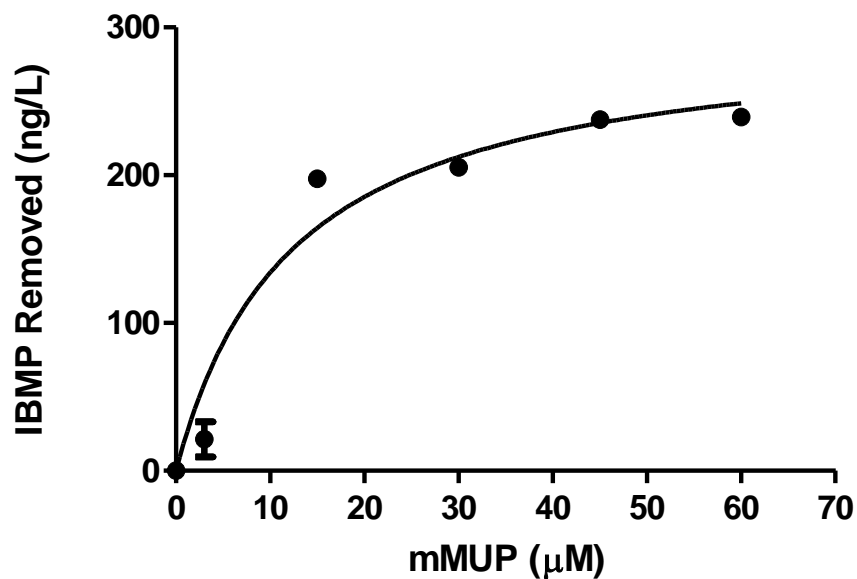
Figure 4.7. Binding of IBMP at 300 ng/L with increasing mMUP concentrations. (A) The free IBMP fraction was measured by GC/MS following separation from protein-bound complex by bentonite fining. (B) The removed IBMP fraction was determined from the difference between the residual IBMP after bentonite fining followed by filtration with no protein addition and the free IBMP after protein addition and removal by bentonite fining and filtration.
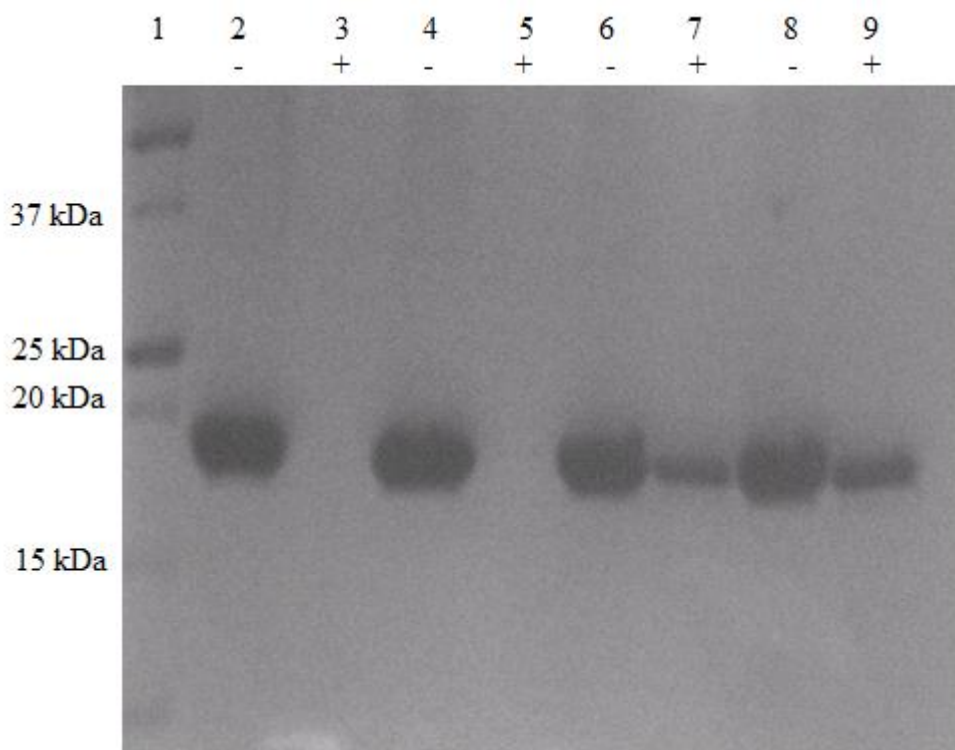
Figure 4.8. SDS PAGE gel of mMUP protein remaining after bentonite fining (3 g/L) and filtration of increasing amounts of protein. Lane 1 represents the Bio-Rad marker, lane 2 is the 15 µM of plOBP with no bentonite fining. Lane 3 represents 15 µM plOBP after bentonite fining. Lane 4 represents 30 µM plOBP with no bentonite fining. Lane 5 represents 30 µM plOBP after bentonite fining. Lane 6 represents 45 µM plOBP with no bentonite fining. Lane 7 represents 45 µM plOBP with bentonite fining. Lane 8 represents 60 µM plOBP without bentonite fining. Lane 9 represents 60 µM plOBP after bentonite fining. Protein concentration increases from 15 to 60 µM, and the "-" indicates no bentonite, while the "+" indicates 3 g/L bentonite used.

Based on this data and trying to maximize protein use in the assay while still using a commercially viable concentration of bentonite for protein removal, approximately 40-50 µM of protein would be tested in future binding assays for the MP removal system, as at this concentration, protein could bind to most of the IBMP and still be removed from the reaction via the bentonite and filtration system.

4.4. Removal of MPs by Proteins from Buffer and Juice Matrix

In order to test the application of the protein to remove the methoxypyrazines, it was important to test the ability of proteins to remove the methoxypyrazines from buffer first in order to ensure that removal was obtainable, then to move to a juice matrix to test the application of the system to the wine industry. An example of the chromatogram that is obtained using the GC/MS to quantify methoxypyrazines is shown in Fig. 4.9. In order to quantify the amount of MP being removed from the solution, area under the peak of the ion of interest from the MP was compared to area under the peak of the deuterated ion that was used as a constant and standard. This integrated number would then be implemented into the equation of the line from the standard curve in order to determine how much of the MP was remaining in the solution. The area underneath the peak is calculated and the ratio of ion 124 to ion 127 is what is use to quantify the amount of IBMP in the sample.
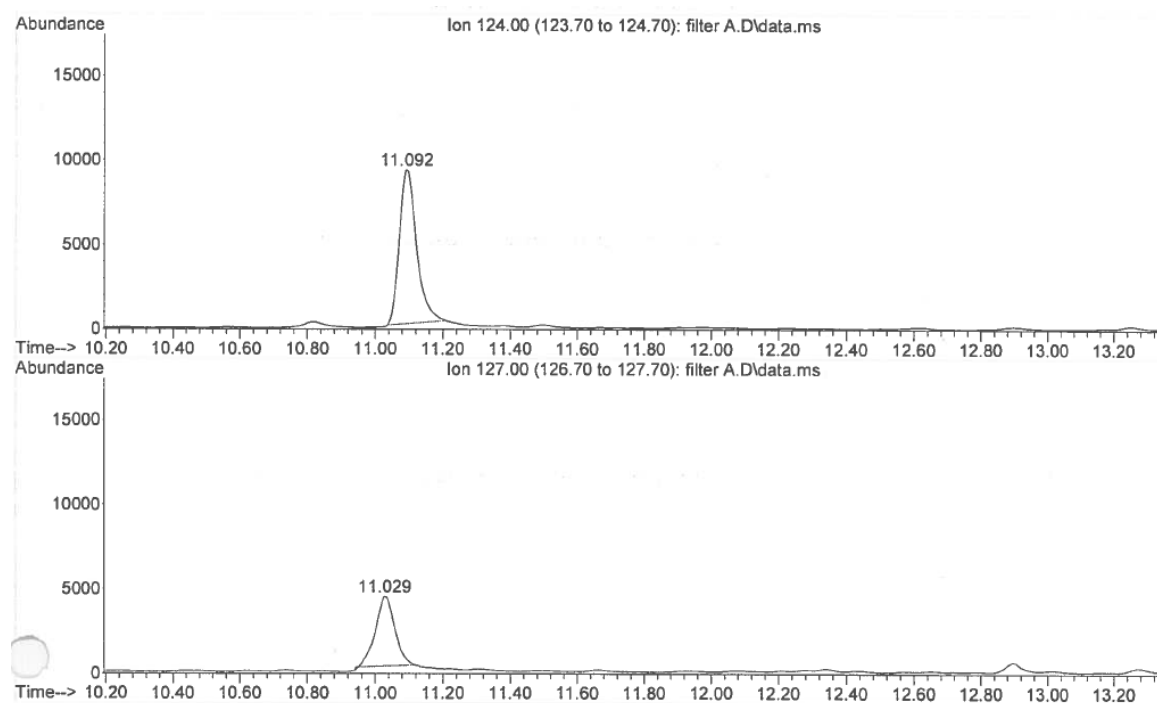
Figure 4.9 GC/MS chromatogram with ion extraction of IBMP at ion 124 and for d-IBMP at ion 127.

The ability of the mMUP protein to remove IBMP from different matrices was shown in Fig. 4.10. The amount of MP removed was determined by the ratio of MP to d-MP. The standard that was used in the determination of binding ability was the juice or buffer spiked with the starting concentration of MP with no treatment done on the solution. The purpose of this was to be able to determine what the starting concentration was for each run on the GC/MS. The purpose of testing filtration alone and bentonite and filtration together, was to be able to determine how much MP could be removed prior to the introduction of protein into the environment. This allowed for an accurate calculation on the amount of MP that the protein would be able to remove itself and the adsorptive effect of the filter and the bentonite on MPs. After the tests were complete in a buffer matrix, they were then performed in a juice matrix. It was important to first test in a buffer in order to ensure that the protein could bind to the MP before testing this in juice. In comparing the ability of the protein to remove IBMP from solution in both pH 4.0 and pH 3.5 buffer, the mMUP was able to remove almost 90% of the total amount of IBMP present in the starting reaction, down to the limit of quantification, which was 6 ng/L. This indicates that mMUP has a high affinity for IBMP in low pH buffer. Next the protein was tested in the juice matrix with sterile filtered Chardonnay juice (pH 3.5). The run in the Chardonnay appeared to show that the mMUP was not able to remove the IBMP down past the limit of quantification, however due to limited quantities of this juice, the experiments could not be further replicated past n = 3, and therefore all future experiments were carried out to an n=6 using sterile filtered Riesling juice (pH 3.5). It is again apparent that the protein was able to remove the IBMP down to the limit of detection.

Figure 4.10 Reduction of IBMP from, Phosphate Citrate Buffer pH 4.0 (n=6), Phosphate Citrate Buffer pH 3.5 (n=6), Riesling Juice pH 3.5 (n=6) and Chardonnay Juice pH 3.5 (n=3) using mMUP protein and the bentonite system. Treatments which were significantly different are represented by different letters. Data analysis was performed using ANOVA and mean separation by Fisher LSD (P<0.05).

Since the causal agent for the Ladybug taint is IPMP, it was important to test the binding affinity of IPMP with mMUP, now that it was shown that mMUP is able to bind to IBMP.

Again mMUP showed that it has the ability to bind to IPMP and remove the chemical down to almost the limit of quantification of 2ng/L (Fig. 4.11).

Figure 4.11 Reduction of IPMP from, Phosphate Citrate Buffer pH 3.5 (n=6) and Riesling Juice pH 3.5 (n=6) using mMUP protein and the bentonite system. Treatments which were significantly different are represented by different letters. Data analysis was performed using ANOVA and mean separation by Fisher LSD ($P<0.05$).

plOBP was the initial protein of interest and was the protein with which the studies were first done on, however the protein did not function as well as had been expected. Initial binding studies (data not shown) showed the protein was not capable of removing IBMP at pH 3.0 beyond that which had already been removed by bentonite and filtration, therefore buffer pH were increased to pH 3.5 and pH 4.0 for subsequent binding studies. At these two pH levels, the ability of the plOBP to remove IBMP was not hindered however the protein was only able to remove a small additional amount of IBMP compared to the bentonite fining system, with approximately 100 ng/L of the IBMP remaining. As in the juice sample the protein was not able to remove a significantly different amount of IBMP compared with what was removed by the bentonite treatment followed by filtration.

Figure 4.12 Reduction of IBMP from, Phosphate Citrate Buffer pH 4 (n=12), Phosphate Citrate Buffer pH 3.5 (n=12) and Chardonnay Juice pH 3.5 (n=6) using pIOBP protein and the bentonite system. Treatments which were significantly different are represented by different letters. Data analysis was performed using ANOVA and mean separation by Fisher LSD (P<0.05).

It was apparent that the protein was able to function and remove some of the IBMP in buffers. It was also important to determine how it would work with the removal of the IPMP.

Fig. 4.13 shows that plOBP did not seem to be able to bind to and remove IPMP in neither buffer nor juice at pH 3.5, although it was able to bind to IBMP and remove some from pH 3.5 and 4.0 buffers, with approximately 1/3 of the starting concentration of IBMP in the solution (Fig. 4.12). It was attention grabbing to see that there had been a greater binding affinity with IBMP than with IPMP, so in order to rule out any scepticism as perhaps the results were indicative of using different protein preparations or running the experiments on different days, an extra experiment was performed. In this experiment, protein samples from the same preparation were used and tested on the same day, one for removal of IBMP and the other for removal of IPMP. Figure 4.14 clearly demonstrates that again plOBP had a greater affinity with the IBMP then with the IPMP, which agreed with literature and did confirm that the results obtained in Figure 4.13 were accurate and precise.

In both the buffer and wine, the protein-MP complex was removed from the reaction through the use of bentonite. This was confirmed in each of the trials through SDS gel electrophoresis. In Figure 4.15 and 4.16 the removal of the mMUP-MP complex from the juice matrix can be seen (similar SDS gel electrophoresis figures can be found in the Appendix 2, for removal of both IBMP and IPMP with mMUP from a buffer matrix). This does coincide with the results seen in Figure 4.10 and 4.11, as the amount of MP was reduced to below the detectable level. The results did not align as nicely with plOBP and the removal of the MP's from either buffer or juice. As stated from Figure 4.12 plOBP was not able to remove a significantly different amount of IBMP than the filtration and bentonite had in the chardonnay juice matrix, however Figure 4.17 shows that indeed all of the protein was removed by the bentonite. Similarly, as seen in Figure 4.13, the removal of IPMP by plOBP in the Riesling juice was not reduced by the presence of plOBP. Figure 4.18 shows that again all of the protein was

removed by the bentonite (Similar SDS gel electrophoresis figures can be found in the Appendix 2, for removal of both IBMP and IPMP with plOBP from a buffer matrix). It is important to ensure the removal of the proteins, in order to ensure that the residual MPs measured in the reaction were not because of an inability to remove the protein-MP complex from the solution.

Figure 4.13 Reduction of IPMP from Phosphate Citrate Buffer pH 3.5 (n=6) and Riesling Juice pH 3.5 (n=6) using plOBP protein and the bentonite system. Treatments which were significantly different are represented by different letters. Data analysis was performed using ANOVA and mean separation by Fisher LSD (P<0.05).

Figure 4.14 Comparison between reduction of IBMP and IPMP from Phosphate Citrate Buffer pH 3.5 (n=3) using plOBP protein and the bentonite system. Treatments which were significantly different are represented by different letters. Data analysis was performed using ANOVA and mean separation by Fisher LSD (P<0.05).
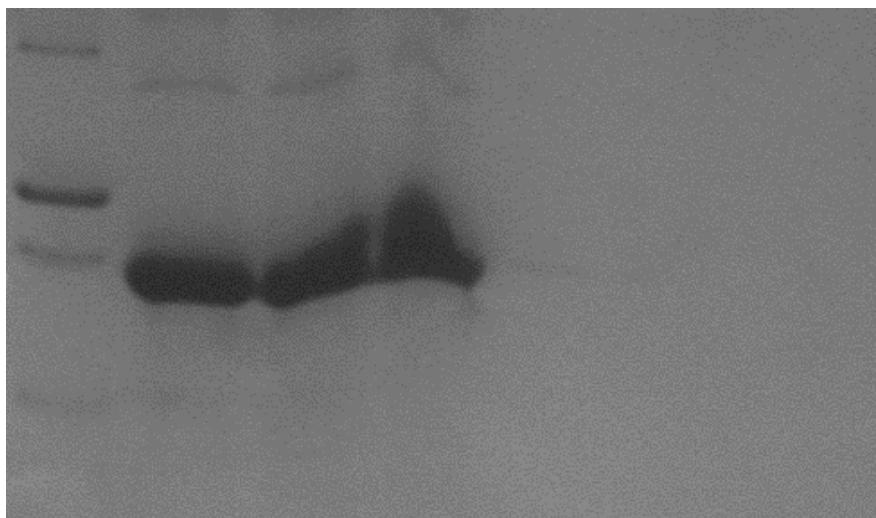
Figure 4.15. Bentonite removal of mMUP-IBMP complex from Riesling juice matrix. Lane one represents the Bio-Rad marker , lanes 2-4 is the juice matrix with mMUP before bentonite removal. Lanes 5-7 are the juice matrix after bentonite removal of mMUP
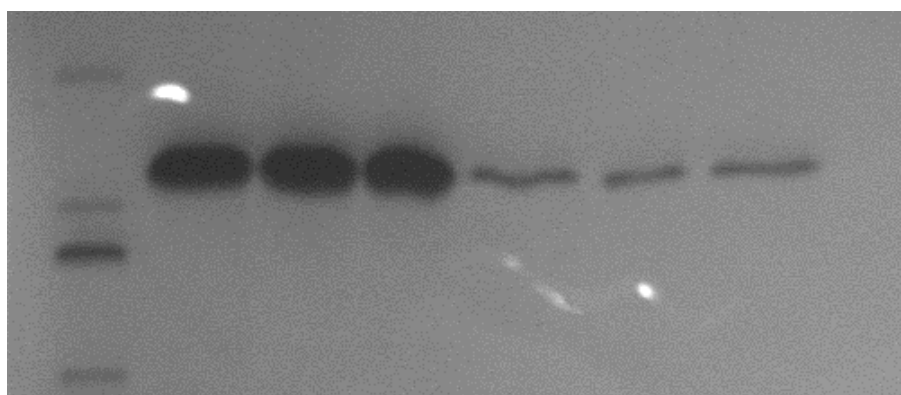
Figure 4.16. Bentonite removal of mMUP-IPMP complex from Riesling juice matrix. Lane one represents the Bio-Rad marker , lanes 2-4 is the juice matrix with mMUP before bentonite removal. Lanes 5-7 are the juice matrix after bentonite removal of mMUP
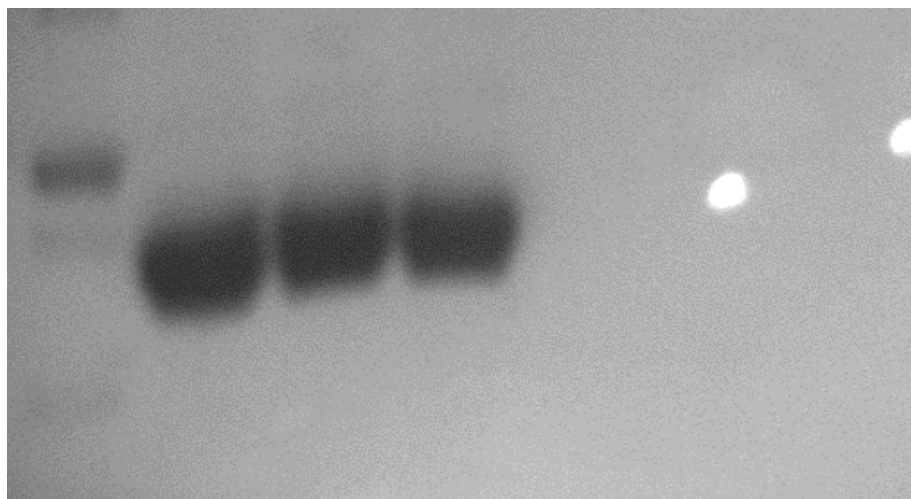
Figure 4.17. Bentonite removal of plOBP-IBMP complex from Chardonnay juice matrix. Lane one represents the Bio-Rad marker , lanes 2-4 is the juice matrix with mMUP before bentonite removal. Lanes 5-7 are the juice matrix after bentonite removal of mMUP
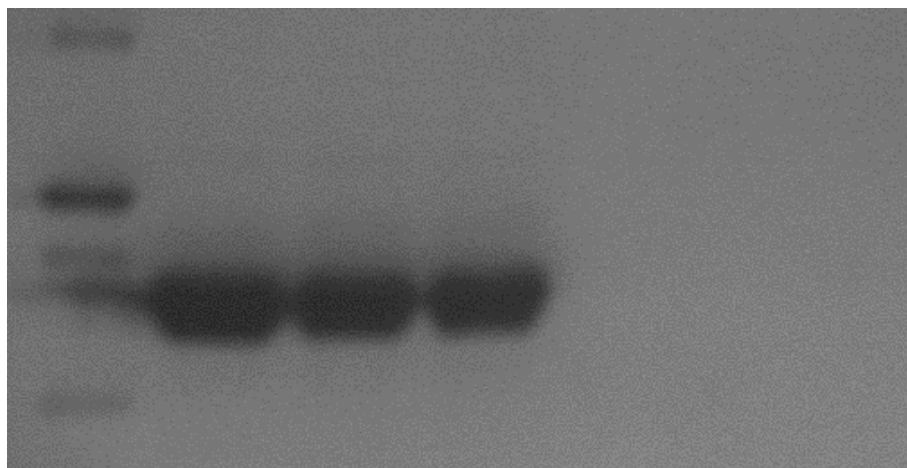

Figure 4.18. Bentonite removal of mMUP-IPMP complex from Riesling juice matrix. Lane one represents the Bio-Rad marker , lanes 2-4 is the juice matrix with mMUP before bentonite removal. Lanes 5-7 are the juice matrix after bentonite removal of mMUP

5. Discussion

5.1 Growth of *P. pastoris* Transformants, and Expression of the plOBP and mMUP

The transformants were first grown up in a glycerol buffered media, in order to accumulate cell mass but not express the protein, as the presence of glycerol inhibits the expression. Expression was not able to occur until after the cells had been spun down and re-suspended in methanol media. Methanol is required for full induction to occur. By allowing the cells to grow in the glycerol environment, it allowed the cell mass to be enhanced, so that when induction was triggered, the amount of protein that could be produced was greater, due to the increase in cell mass. This is common practice in order to obtain the greatest amount of the recombinant protein (Sudbery, 1996). There was no expression observed on the day of inoculation however on the subsequent days that followed expression did occur, with the day two of expression resulting in the greatest amount of protein being expressed. It had been determined in earlier research (Humes, 2008 unpublished) that after the second day of expression the protein concentration started to decrease. Each day a sample of the induction media was collected and Bradford assays were run in order to quantify the amount of protein being expressed. Both the mMUP and plOBP had significant increases in protein expression by the second day of expression.

Growth was done under a controlled environment where the temperature never exceeded 29°C; this is optimal for the growth of yeast, as this temperature increases growth and survival for the yeast (Heard and Fleet, 1988).

S. cerevisiae is often limited as an expression system by low yields; however the methylotrophic yeast *P. pastoris* retain all the advantages of *S. cerevisiae* but provide a reliable means of achieving greatly elevated yields (Sudbery, 1996).

The *P. pastoris* was able to act as a perfect expression system for the expression of the recombinant proteins from the higher eukaryotes (plOBP and mMUP). The simplicity and ease, was very similar to using a bacterial expression system, with the authenticity of the less convenient animal tissue culture systems (Sudbery, 1996). It is the fact that the yeast provide an environment for post-translational processing and secretion, which it is able to allow for the expression and secretion of proteins that appear to be in the native form.

5.2 Purification of Recombinant Proteins

The expressed protein that was collected at day 2 of expression was dialysed in a pH 8.0 buffer in order to increase the pH of the expressed proteins as they were induced in a pH 6.0 environment. Dialysis was also done in the 10kDa MWCO tubing in order to remove any degraded proteins, as well as other possible expressed proteins and smaller compounds that fit through the tubing, this just lessens the amount of different solutes being passed through the FPLC column and perhaps interfering with the proteins being bound to the negatively charged resin in the column. After the four days of dialysis, the protein samples are run through the FPLC in order to remove any unwanted proteins and peptides. The recombinant protein of interest is eluted from the column as the salt concentration increased to between 40 to 70%. The protein that is eluted was pooled together and dialysed in MilliQ water, in order to try and remove the salt, so that the salt would not play a factor in the function of the protein. After dialysis the purified protein is aliquoted out and freeze dried. Bradford results indicated that the

overall protein recovery from start to finish was 78% for plOBP and 62% for mMUP, suggesting that the *P. pastoris* expression system is very efficient when expressing the recombinant proteins of interest. This was to be expected as the proteins of interest were integrated into the gene cassette of the *P. pastoris* so that the expressed protein would be excreted from the cells by the yeast secretary system, and these proteins could then be collected and purified so to increase the concentration of the protein in solution.

5.3 Removal of Protein from System

When fining proteins from wine or juice, it is of general practice to use bentonite as a fining agent to remove the proteins (Blade and Boulton, 1988); this was the basis of the decision to use bentonite to remove the recombinant proteins from solution. The 3g/L of bentonite was an adequate amount of bentonite to remove the protein and within the concentration range used commercially in wineries. In common wine fining practice, the amount of bentonite used varies from 1-3 g/L (Blade and Boulton, 1988), therefore we were able to perform the experiments while staying in the range of appropriate amounts of bentonite. Bentonite is capable of removing more than just the protein of interest, and thus it is important to not use too much bentonite as the effects would be unknown, until taking the juice/wine solution to a sensory lab in order to determine what detrimental effects the bentonite had on the juice. In order to determine that 3g/L was adequate the experiments were run where the amount of the bentonite was increased until all of the protein was removed. This was determined by running the samples on SDS PAGE gels and performing Bradfords. It would be interesting to see if 2 g/L would have been adequate as well, as only 1, 3, 5, and 7 g/L were tested. Also of interest would be to test various types of commercial bentonite preparations to investigate the strength of the various amounts of

bentonite with varying concentrations of protein. Future optimization of the system is still warranted.

5.4 MP-Protein-Bentonite Binding Complex

Testing the ability of the protein to remove the methoxypyrazines was done once the optimal concentration of protein was determined through the use of increasing the concentrations in order to determine if saturation could be reached, this would allow no protein to be wasted as only the optimal concentrations would be used. Although saturation was never achieved when testing from 0 – 60 µM of protein, it was determined that using approximately 40 µM of protein was optimal.

mMUP protein was capable of removing the IBMP and IPMP by almost 95%, regardless of whether tested in pH 3.5, 4.0 or in the Riesling juice pH 3.5. The protein however was not able to remove the same amount of MP from the Chardonnay juice. This could be due to the difference in the chemical composition of the two juices, as Riesling juice is known to have different volatile compounds than Chardonnay juice does (Dumont *et al.,* 1994). The volatile compounds which are found more frequently in Chardonnay could be more susceptible to binding with the protein, thus reducing the amount of MP which would be removed. Protein was successfully removed from these test reactions by the bentonite, as this was confirmed through SDS PAGE gels, and Bradford analysis. mMUP removed a significantly different amount of MPs than the bentonite was able to. Each of the steps of the binding reactions was significantly different in their ability to remove MPs.

plOBP was also capable of removing IBMP but only by about 30% of the starting concentration, and it was not successful in removing IPMP from the solution. Part of the reason

for this difference between IBMP and IPMP is likely due to the fact that OBP's are known to have a greater affinity for IBMP than IPMP, which was indicated by Briand *et al.,* in 2000, However, additional studies using plOBP from the Inglis laboratory, summarized in the patent application in appendix 1 (Inglis *et al*., 2010) have shown that plOBP is able to remove both IPMP and IBMP when the protein-MP complex is removed using a membrane filtration assay that traps the protein-MP complex. It is likely that bentonite may be binding to plOPB and altering the binding pocket, changing the affinity of plOBP to MPs and/or increasing the off-rate of MPs from the protein binding pocket. As the bentonite binds to the plOBP, this could lead to an alteration of the protein conformations and perhaps adsorption (Blade and Boulton, 1988). Another possibility is that the ability for the bentonite to bind to the plOBP was affected by competition from other cations in the solution. The bentonite can cause a disruption of the ability for the protein to absorb the MPs, due to the presence of sodium in the solution from the bentonite, can lead to a decreased absorption of the MP with the plOBP, however the bentonite will have a greater affinity for the protein (Blade and Boulton, 1988). Conformation changes in the protein lead to some unfolding, which will result in an increased number of protein sites contacting the surface of the bentonite and thus this would affect the active site of the protein which would have bound to the MP, potentially releasing the MP from the pocket (Israel, 2009). There is a chance that the conformational differences between the Odorant and the Pheromone Binding Proteins, is what lead to the plOBP not being able to bind to and remove the same amount of MP as the mMUP had in the presence of bentonite, but why in other research done in the Inglis Lab both proteins were shown to be able to bind and remove the MP when used with a membrane. For the bentonite removal system for the protein-MP complex, results are indicating that plOBP was not as ideal of a protein fining agent as the mMUP.

5.5 Future Research

It would be of interest to investigate the binding ability of the mMUP protein at the pH 3.0, as the plOBP protein was not capable of significantly removing more MPs then the bentonite did, however with the mMUP protein being able to remove such a significantly greater amount of MPs perhaps it would still be able to function at the more acidic pH, as the general pH of juice is between 3.0-4.0, so it would be of importance to investigate the lower pH. Also of interest is the ability for the protein to function in the presence of ethanol, as ethanol can affect the binding ability of proteins, however the determination of whether or not the protein could function in the presence of ethanol, would allow these proteins to be used for MPs removal in wine. It was noted in the saturation curves of the mMUP that indeed saturation occurred at 30 μM of the protein, so perhaps trying the binding assays, using a lesser amount of mMUP would allow one to run more experiments do to having to use less protein, this would ensure that the reason mMUP worked so well as a fining candidate was not because there was too much protein used, but because it has a greater binding affinity for the MPs.

It is necessary to try and scale up the amount of protein being produced and the effect it will have on larger volumes of juice, therefore determining a way to perhaps reuse the protein would be of interest. It would also be of great value to develop a commercial application for this technology by covalently binding the protein to an inert support such that the juice and/or wine is passed over the support to remove MPs from solution. The proteins could be bound to silicon dioxide particles or a polyethersulfone membrane such that the protein is never free in the wine solution. Silicon dioxide particles with the protein, either on the surface or in the interior of the particle, could be easily applied to or removed from juice using apparatus in the wineries already

in common use for bentonite fining. A protein-coated membrane could simply remove MPs through filtration. Both methods are of interest to be developed as a multiple use system.

It is still of great importance to investigate what effects the protein fining and bentonite fining has on the finished wine product. Although the proteins studied in this project have high affinity to MPs, the specificity in a juice matrix has not yet been investigated. Taking the fined juice and fermenting it to produce wine, and then performing chemical and sensory analysis on these wines will ascertain if the MP removal system has any detrimental effects of flavour compounds in the wine.

6. Conclusion

The yeast expression system was successful at expressing plOBP and mMUP in buffered methanol media, and reached a peak of protein concentration over a two-day expression.

Purification of plOBP and mMUP was capable of concentrating the protein and leading to a percent recovery of 78% and 62% respectively, this indicated that protein expression was efficient and that anion exchange was successful in concentrating and purifying the protein.

It was determined that 3g/L of bentonite was sufficient to remove both proteins from the solution at both pH 3.0 and 4.0, keeping in proper practice, of wine fining.

plOBP was capable of binding to IBMP but was not capable of binding to IPMP in either matrix. Therefore it is not an ideal protein to be used as fining agent as it was not able to always significantly remove MPs below what the bentonite was capable of removing.

mMUP successfully removed both MPs from Riesling juice and buffer to the limits of quantification, in any environment it was tested in, it is the ideal candidate for future studies in fining MPs out of juice

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Appendix 1 – Patent application

Appendix 2 – SDS - Gel electrophoresis of protein – MP complex in buffer before and after bentonite fining.
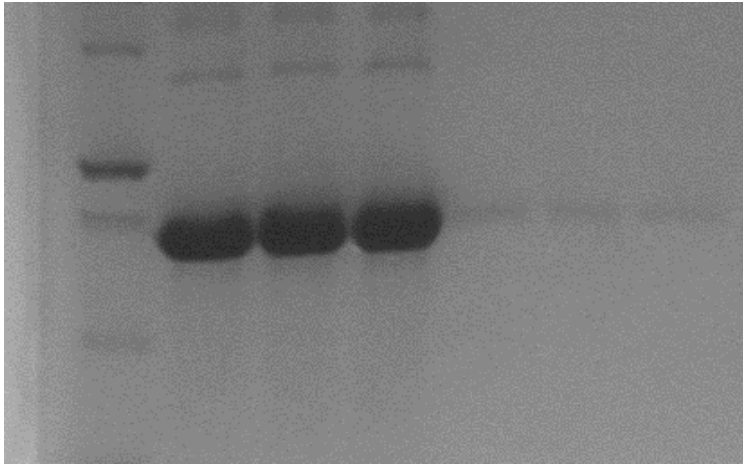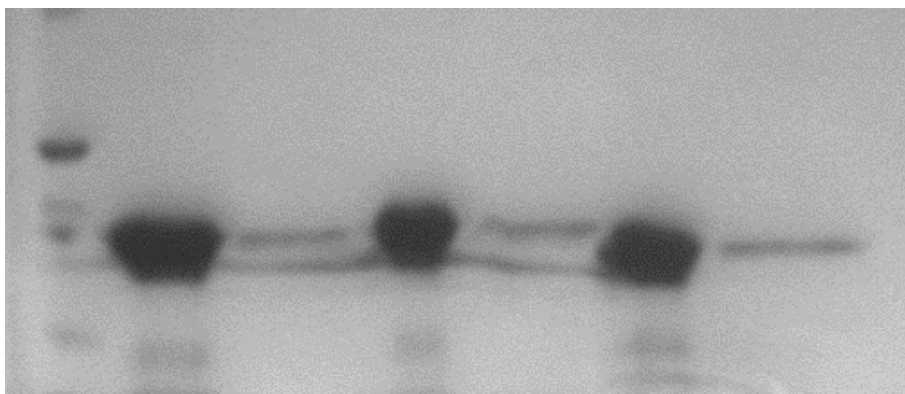

Figure A1. Bentonite removal of mMUP-IBMP complex from 3.5 pH buffer. Lane one represents the Bio-Rad marker , lanes 2-4 is the juice matrix with mMUP before bentonite removal. Lanes 5-7 are the juice matrix after bentonite removal of mMUP
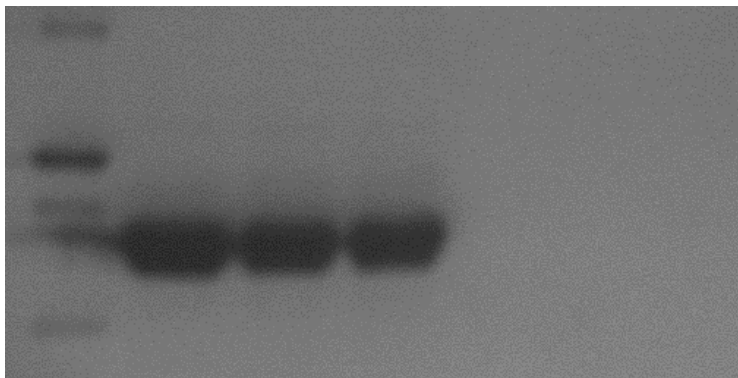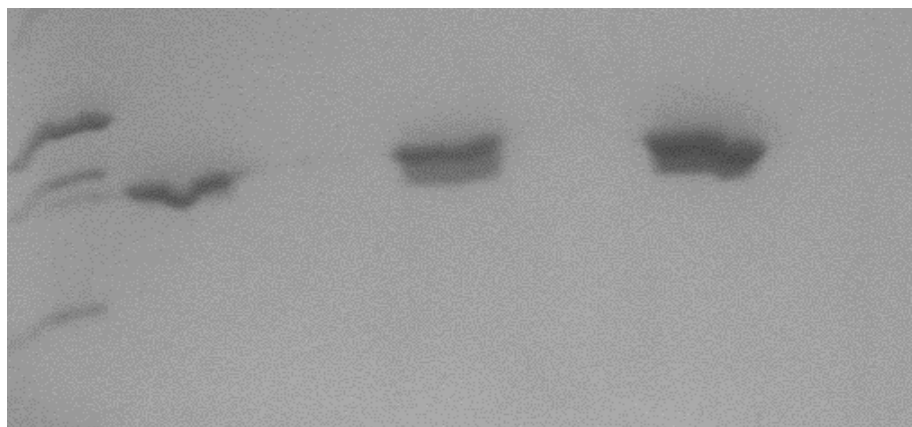

Figure A2. Bentonite removal of mMUP-IBMP complex from 4.0 pH buffer. Lane one represents the Bio-Rad marker , lanes 2-4 is the juice matrix with mMUP before bentonite removal. Lanes 5-7 are the juice matrix after bentonite removal of mMUP
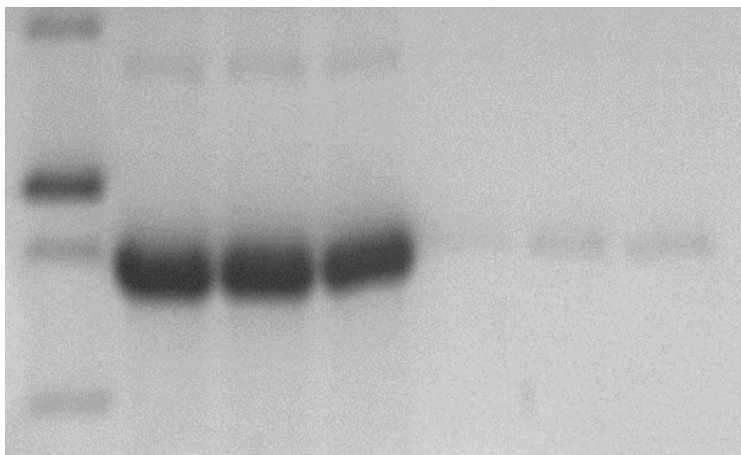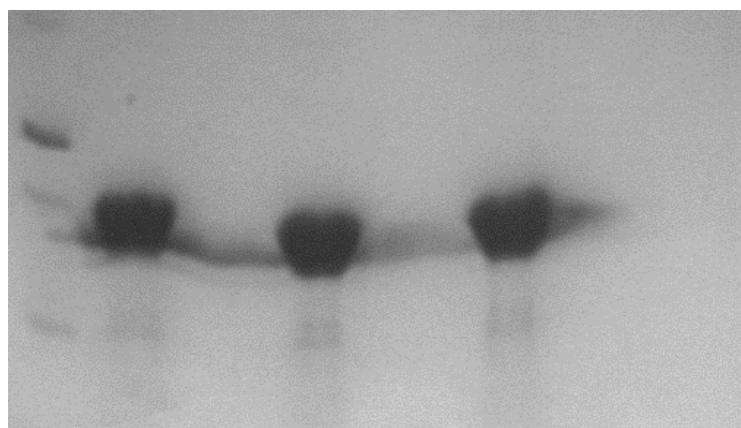
Figure A3. Bentonite removal of mMUP-IPMP complex from 3.5 pH buffer. Lane one represents the Bio-Rad marker , lanes 2-4 is the juice matrix with mMUP before bentonite removal. Lanes 5-7 are the juice matrix after bentonite removal of mMUP

Figure A4. Bentonite removal of plOBP-IBMP complex from 3.5 pH buffer. Lane one represents the Bio-Rad marker , lanes 2, 4 and 6 is the juice matrix with plOBP before bentonite removal. Lanes 3, 5 and 7 are the juice matrix after bentonite removal of plOBP

Figure A5. Bentonite removal of plOBP-IBMP complex from 4.0 pH buffer. Lane one represents the Bio-Rad marker , lanes 2, 4 and 6 is the juice matrix with plOBP before bentonite removal. Lanes 3, 5 and 7 are the juice matrix after bentonite removal of plOBP

Figure A6. Bentonite removal of plOBP-IPMP complex from 3.5 pH buffer. Lane one represents the Bio-Rad marker , lanes 2,4 and 6 is the juice matrix with plOBP before bentonite removal. Lanes 3, 5 and7 are the juice matrix after bentonite removal of plOBP