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The Use of Thaumatin and Bovine Serum Albumin as Proteins in Model Wine Solutions in Bentonite Fining

This study examined the viability of using thaumatin and bovine serum albumin (BSA) as proteins in model wine solutions for bentonite fining studies and compared them with unfined New Zealand sauvignon blanc (SB) wine. Bentonite fining trials were performed on model wine solutions and unfined SB wines (pH range 3.5 to 4.3). Thaumatin was more readily adsorbed onto bentonites of all types than BSA and its adsorption onto bentonite was less affected by the pH of the solution. Specifically, the amount of BSA adsorbed onto bentonite decreased significantly as the pH of the solution approached the isoelectric point (pI) of BSA while thaumatin was adsorbed at that pH due to its higher pI. Changing pH affected protein adsorption of real wine less noticeably than of BSA and thaumatin, and decreasing pH increased protein adsorption in contrast to the model solutions. Neither of the model solutions can fully represent the response of real wine to bentonite fining but they are simple and cost effective to prepare and reacted to changes in bentonite concentration similar to real wine. Thaumatin is potentially a better protein to use in simple model solutions for wine stabilisation studies like filtration where molecular weights are important consideration.

Keywords: thaumatin; bovine serum albumin; bentonite fining; model wine; proteins

Introduction

Bentonite fining is a process to remove proteins from wines to ensure protein stability. The biggest contributors to protein instability were discovered to be pathogenesis-related (PR) proteins, thaumatin-like proteins and chitinases (Hayasaka et al., 2001; Hsu & Heatherbell, 1987a; Waters, Wallace, & Williams, 1992). There are many studies on the mechanisms of bentonite fining and investigations related to improvements, refinement or the replacement of the bentonite fining process (Lambri, Dordoni, Giribaldi, Violetta, & Giuffrida, 2012; Pocock, Salazar, & Waters, 2011; Waters & Colby, 2009).

Since wines are complex solutions of various compounds, simplified *model* wine solutions are frequently used by researchers to control the number of variables when designing new techniques to process wine where these solutions are used to predict the effectiveness of the techniques (Blade & Boulton, 1988; Sun et al., 2007; Lagace & Bisson, 1990; Pocock, Høj, Adams, Kwiatkowski, & Waters, 2003; Tran, Patterson, & James, 2012). A commonly used protein in model wine solutions is bovine serum albumin (BSA) because it is well researched and easily available (Blade & Boulton, 1988; Marchal, Seguin, & Maujean, 1997; Murphey, Powers, & Spayd, 1989; Tran et al., 2012). However, BSA may not be an acceptable protein to use in the study of new fining processes where molecular weight is an important factor.

Wine proteins' molecular weight range is between 11.2 and 190kDa (Hsu & Heatherbell, 1987b; Somers & Ziemelis, 1973) with isoelectric points (pI) between 2.5 and 8.7 (Anelli, 1977; Dawes, Boyes, Keene, & Heatherbell, 1994). The major protein fractions found in wine haze are the 24kDa (thaumatin-like) and 32kDa (chitinases) (Waters, Shirley, & Williams, 1996). Subsequently, chitinases had been found to be the most important protein fraction in haze formation (Falconer et al., 2010; Marangon et al., 2011). The ideal protein for a model wine solution should then similar to the haze-causing proteins, particularly in the properties important to the techniques being studied, and remain available and affordable.

This study examines thaumatin (pI of 12 and molecular weight between 20 to 22kDa) as a protein option in model wine solutions (van der Wel & Loeve, 1972). This is compared against BSA (pI of 4.7 or 4.9 and molecular weight of 66.4kDa), which is within the range of wine proteins' pI, but almost triple the molecular size of thaumatin-like proteins and double the size of chitinases (Malamud & Drysdale, 1978; Hirayama, Akashi, Furuya, & Fukuhara, 1990; Hayasaka et al., 2001).

Thaumatin-like proteins have acidic isoelectric points in white wines (Liu, Sturrock, & Ekramoddoullah, 2010; Bayly & Berg, 1967; Hsu & Heatherbell, 1987b). While thaumatin and thaumatin-like proteins are not entirely the same, they have very similar molecular weight ranges, as well as both being positively charged at wine pH levels, with significant similarities between the homologies of these proteins, despite thaumatin-like proteins having an acidic isoelectric point (Liu et al., 2010).

It is known that different proteins respond differently to bentonite adsorption (Achaerandio, Pachova, Güell, & López, 2001). This study examines the viability of using the commercially-available thaumatin as a cost effective model wine component. This particular model wine would be most appropriate as a screening tool used in investigating fining processes where protein separation by molecular weight is relevant. The results presented in this study, therefore, examine and compare both BSA and thaumatin as proteins in model solutions, and compare their performance to that of real wine during bentonite fining. The aim of this study was to determine whether thaumatin is a satisfactory model solution component in predicting bentonite adsorption of proteins in wine.

Materials and Methods

Sample Solutions

BSA (MP Biochemicals) and thaumatin (kindly donated by Natex UK) were the proteins used. The proteins were added to a 12% ethanol-water solution then stirred gently for an hour to form a 0.1g/L solution, similar to the protein concentration in the unfined wine samples. A 2g/L tartaric acid solution was used to adjust the pH (3.5, 4.0 and 4.3) of the final model solutions. Unfined sauvignon blanc (supplied by Pernod

Ricard Winemakers, New Zealand) was used as comparison, its pH adjusted to match those of the model wine solutions using tartaric acid and potassium carbonate.

Fining Tests

Each of the two model wine solutions was used in fining trials at three different pH conditions, and using three different bentonites at three different bentonite concentrations each. The three bentonites used were: laboratory grade pure bentonite (high purity montmorillonite from Sigma-Aldrich, designated Bentonite A), Aktivit® and Seporit® (designated Bentonites B and C respectively, both sodium-calcium bentonites by Erbslöh, supplied by Pernod Ricard Winemakers, New Zealand). Bentonites B and C are commercially sold bentonites marketed for wine and juice fining. The hydrated particle size distribution of the bentonites was examined using a particle size analyser, Malvern Mastersizer2000. 5% bentonite solutions were made with deionised water, stirred gently for 24 hours before use. Fining tests were performed using 0.1, 0.2 and 0.3g/L (bentonite to sample volume) for bentonite A, and 0.4, 0.6 and 0.8g/L for bentonites B and C. The difference was due to the higher adsorption capacity of Bentonite A as discussed later in the Results. The solutions were mixed gently followed by a settling period of 8 hours. The bentonite was then removed by centrifugation (10000g for 10min) before analysing protein concentration and stability.

Protein Concentration Determination

Protein concentration of the model solutions was determined using a Bradford assay kit (Coomassie Plus, Thermo Fisher Scientific). The analysis method was modified from the manufacturer's recommendations to improve sensitivity at the tested protein concentration range. Bradford solution was added to samples in 2:1 volume ratio and then measured for absorption at a wavelength of 595nm using UV-Vis spectroscopy

after a 10 minute incubation time. Previous studies had established that protein concentration in wine is not an accurate measurement for its heat stability (Bayly & Berg, 1967; Moretti & Berg, 1965). Wine makers still rely on stability tests for this purpose (Esteruelas et al., 2009; Toland, Fugelsang, & Muller, 1996), and one of such tests is the Prostab analysis kit (purchased from Fleurieu Winery Supplies), a commercial wine analysis kit previously found to be a rapid analysis that generated results very similar to other known stability tests by precipitating nearly all dissolved proteins (Esteruelas et al., 2009). This method was used to compare against the Bradford results for wine. Prostab reagents were added to samples in a 1:10 volume ratio and then measured for absorption at wavelength of 600nm using UV-Vis spectroscopy after a 10min incubation time. Standard curves for both tests were prepared for all tested pH values and results of the fining tests are expressed in percentage protein left in the solution after fining.

Statistical Studies

All experiments were performed three times to account for experimental variation. The data was averaged with the sample standard deviations calculated. Analysis of variance (ANOVA) with 95% confidence intervals was also performed on the data using SPSS Statistics software.

Results and Discussions

The standard curves for both Bradford (Figure 1A) and Prostab (Figure 1B) were similar to those provided by the manufacturers. Prostab has greater linearity (linear correlation $r^2 = 0.99$) but less sensitivity than the Bradford assay. Within the pH range used in this study, there was negligible effect of pH on measurements. The two techniques are known to function differently, where the Bradford assay involves

proteins binding to the Coomassie brilliant blue dye resulting in a colour change (Boyes, Strübi, & Dawes, 1997; Bradford, 1976), while Prostab measures protein instability by precipitating dissolved proteins (Esteruelas et al., 2009).

Figure 2 shows that both proteins displayed similar, non-linear trends with increasing bentonite concentrations with all bentonite types, where adsorption was low at very low concentrations, increasing significantly upon a certain threshold before tapering off, similar to the results of a previous study (Hung, 2010). This suggests that the adsorption mechanisms of protein onto bentonite are independent of the type of bentonite used or the protein in question. This figure also shows that thaumatin displays larger reductions in protein concentration with increasing bentonite concentration meaning that thaumatin is more readily adsorbed onto all types of bentonite than BSA at all pH values and the difference is statistically significant ($F(1, 141) = 1842.41$, $p < 0.05$).

Bentonite A was shown to be a significantly more efficient fining agent than the other two bentonites, requiring half the concentration (0.3g/L compared to 0.6g/L) to fully remove proteins under the best conditions (maximum adsorption difference between bentonite B and C within the error of one standard deviation). The differences between the bentonites can be explained by the different hydrated particle size distribution (average d(0.5) of 4.49μm, 8.63μm and 8.17μm for Bentonites A, B and C respectively), where Bentonite A has higher surface area due to the smaller particle size. The similar results with Bentonites B and C were not surprising due to the similar particle sizes and being the same type of bentonite (sodium-calcium). A small difference in the adsorption performance within the same type of bentonite under different labels had been previously reported (Lambri et al., 2012).

Regardless of the bentonites used, both proteins responded to changes in acidity of the model wine solutions with significant statistical difference ($F_{(1, 141)} = 88.76$, $p < 0.05$). The optimal pH of the BSA model solution appears to be 4.0, achieving 94% removal with Bentonite A at 0.3g/L and 63% and 69% with Bentonites B and C respectively at 0.8g/L. BSA was affected to a higher degree by changes in pH ($F_{(2, 141)} = 96.04$, $p < 0.05$). BSA adsorption became negligible when pH increased to 4.3, managing at best a 17% removal. This is likely due to the solution pH being close to the pI of BSA reducing its net charge and thus its ability to adsorb onto bentonite (Boulton, 1998; Marchal & Jeandet, 2009). Thaumatin adsorption showed a preference towards higher pH, different to what was expected, where a lower solution pH should increase the net charge of thaumatin and thus increase its adsorption by bentonite (Marchal & Jeandet, 2009). This is likely caused by the larger amounts of acidity modifying compounds used to achieve the solution pH interfering with bentonite adsorption.

Figure 3 showed the difference between the Bradford assay and Prostab kit results on the fining tests on wine. The results are different since the two techniques work differently. Interfering compounds in wine like phenolics reduce the accuracy of the Bradford assay (Murphey et al., 1989) making it unreliable for wine analysis (Gazzola, Vincenzi, Pasini, Lomolino, & Curioni, 2015). This explains the higher protein concentration measured by Bradford. This makes it necessary to use an alternative method for estimating protein concentration, in this case, using Prostab together with model solution concentration calibrations to derive model solution-equivalent protein concentration and remaining protein as displayed in Figure 3.

Both sets of results showed a dependence of protein removal on the pH of the wine. While both the model wine solutions were affected by pH, neither responded to bentonite fining in the completely same way as unfined wine did. Specifically, for wine,

a lower solution pH increased the level of protein removed via bentonite fining, unlike the model solutions. The highest levels of protein removal for wine were recorded at pH3.5 where 37% of proteins remained (thaumatin equivalent Bradford assay) and got progressively lower as pH increased (64% remaining at pH4.3). In addition, the relationship between bentonite concentration and the amount of proteins removed is more linear for wine than either of the model solutions. These differences are likely caused by the wide range of proteins in real wine (both molecular weight and pI) (Bayly & Berg, 1967; Hsu & Heatherbell, 1987a; Somers & Ziemelis, 1973). The increase in pH reduced the net charge of proteins and their ability to adsorb onto bentonite, but the presence of proteins of higher pI will still be removed at pH4.3. In terms of wine stability, the Prostab test results (Figure 3) highlight that a model wine with thaumatin will underestimate the amount of bentonite required to achieve heat stability while BSA will overestimate.

Neither protein was able to fully duplicate real wine fining, mostly due to the complexity of the 'matrix effect' of wine. Both model solutions will be inaccurate when used to quantitatively predict the effect of bentonite fining but each shows a similar trend to that of real wine. This makes thaumatin a viable alternative to BSA as a protein for model solutions, and a potentially better protein to use in studies of the protein stabilisation process if molecular weights are a crucial consideration (as they would be in membrane filtration). Further studies may include chitinases or chitinases-like proteins as alternatives although cost and availability may become a concern.

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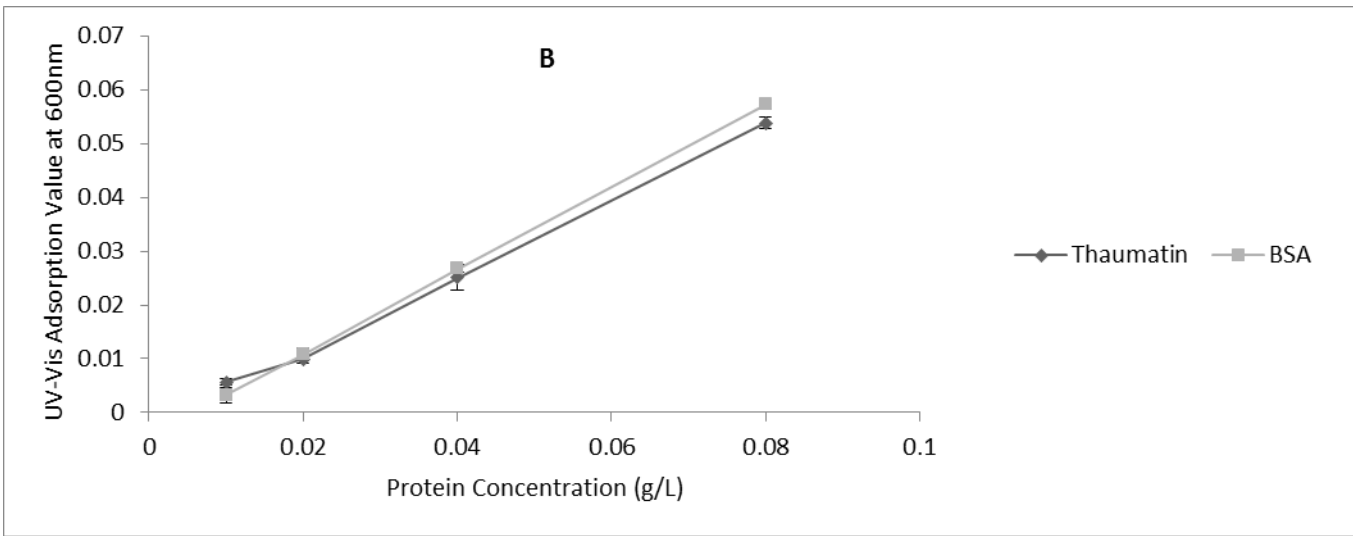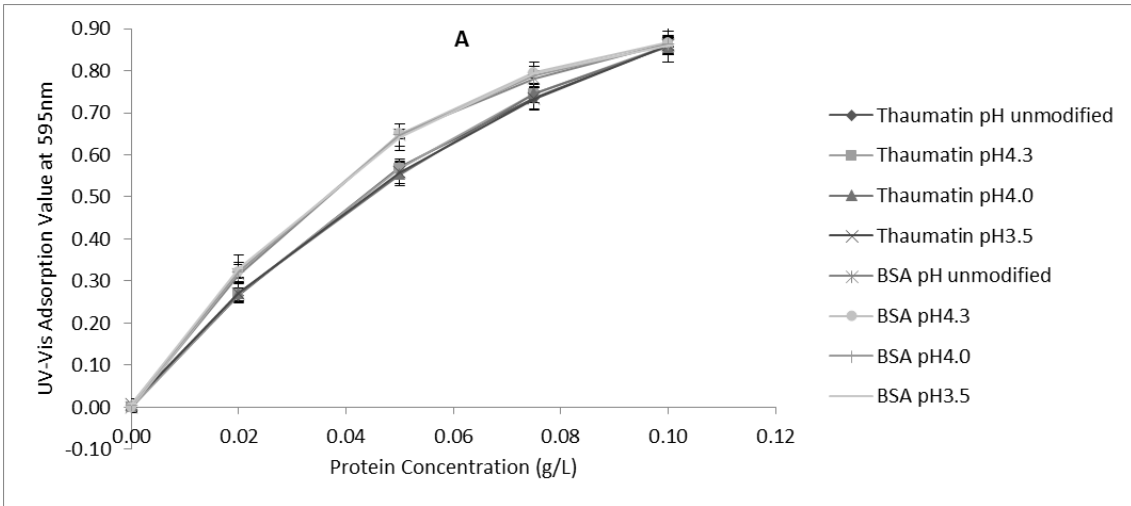
Figure 1: Standard curves for thaumatin and BSA with A: Bradford assay; B: Prostab kit. Error bars indicate 1 standard deviation.

Figure 2: Fining test results on model wine solutions at various pH. A: Bentonite A concentration at 0.1, 0.2 and 0.3g/L; B: Bentonite B concentration at 0.4, 0.6, 0.8g/L; C: Bentonite C concentration at 0.4, 0.6, 0.8g/L.

Figure 3: Percentage protein remaining in sauvignon blanc wine after subjected to bentonite fining using Bentonite A (0, 0.1, 0.2 and 0.3g/L concentrations) as measure by Bradford assay and Prostab kit.

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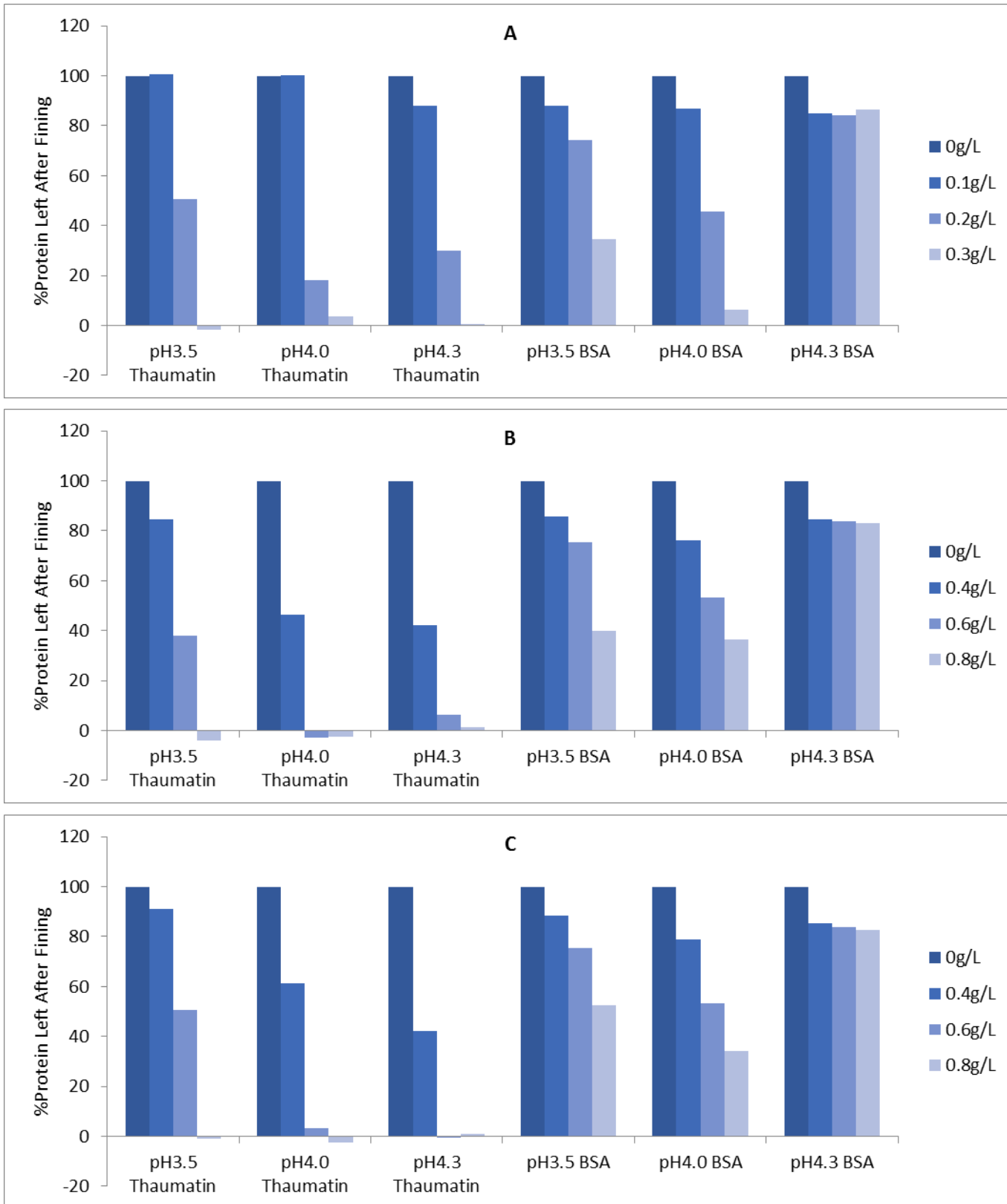
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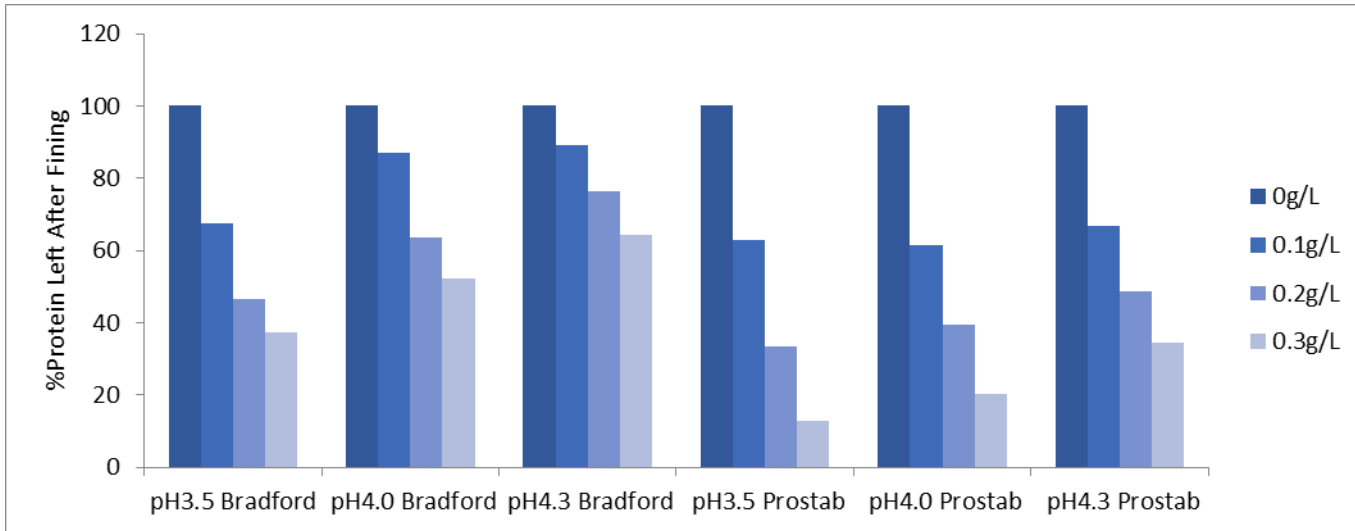
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