TWO PHASE AQUEOUS EXTRACTION OF WHEY PROTEINS IN A POLYETHYLENE GLYCOL – ARABINOGALACTAN SYSTEM

Timothy W.C. Ng[1], Mark C. Lay[1] and Janis E. Swan[1]
[1]School of Engineering
University of Waikato
Private Bag 3105
Waikato Mail Centre
Hamilton 3240
NEW ZEALAND

twcn1@students.waikato.ac.nz

Abstract

The whey protein separation potential of aqueous two-phase systems of arabinogalactan [AG] (Lonza FiberAid™) and polyethylene glycol [PEG], buffered with 10 mmol/g phosphate or citrate buffer, was studied. 100 mmol/g potassium chloride [KCl] was added as required. Previously-published phase equilibrium results were verified and the absorbance of whey protein isolate [WPI] in an AG-PEG solution was measured. The effect of pH, KCl concentration, initial WPI concentrations and upper to lower phase mass ratios on whey partitioning was studied. The best separation system contained 17.20% (w/w) AG, 7.20% (w/w) PEG, 10 mmol citrate buffer (pH 5.4) and 100 mmol KCl per gram of total system. The upper to lower phase mass and volume ratios were 1:1 and 16:11 respectively. Approximately 12 mg (mainly α-lactalbumin) of the 20 mg WPI added partitioned into the AG-rich upper phase. This system has potential to reduce chromatographic requirements in large scale separation of protein mixtures.

Keywords: two-phase aqueous extraction; whey; arabinogalactan; polyethylene glycol

Introduction

Biopharmaceutical production is a rapidly growing industry, and is likely be worth approximately 1.3 trillion USD by 2020 (Rosa *et al.*, 2010). Monoclonal antibodies are an important growth area in the biopharmaceutical industry, and are now estimated to be worth 50 billion USD per annum (Rosa*, et al.*, 2010). Other growth areas include therapeutic recombinant proteins and nucleic acid based medical products. They are all highly effective in their applications, which include treating cardiovascular disease, cancer, autoimmune disorders, inflammatory problems, neurological problems, as well as being used for vaccination purposes (Rosa*, et al.*, 2010).

Unfortunately the cost of producing many biopharmaceuticals remains a barrier to their widespread use. For example, the cost of a monoclonal antibody treatment can be up to 40 thousand USD per patient (Rosa*, et al.*, 2010). Downstream processing costs make up approximately 80% of the total production cost of many biopharmaceuticals, and purification costs make up approximately 70% of the downstream processing costs (Azevedo *et al.*, 2009a; Rosa*, et al.*, 2010).

The relatively high downstream processing costs in biopharmaceutical production are largely due to the reliance on packed bed chromatography at the purification step. Although a mature technology, increases in packed bed chromatography yields have not kept pace with increases in upstream process yields. Hence the cost of packed bed chromatography has increased relative to upstream processing costs (Rosa*, et al.*, 2010). Furthermore, packed bed chromatography technology has a low throughput, high residence time, requires expensive media and is limited to batch operation (Azevedo*, et al.*, 2009a; Rosa*, et al.*, 2010). These all limit the potential for cost reduction.

One way to reduce downstream processing costs is to reduce or eliminate the use of packed bed chromatography at the purification step. This can be done by using complementary technologies such as two-phase aqueous extraction systems. Examples include: using phosphate buffer and polyethylene glycol [PEG] (modified with glutaric acid) to extract monoclonal antibody IgG1 from Chinese hamster ovary (CHO) supernatant (Azevedo *et al.*, 2009b); using PEG and maltodextrin to extract whey proteins (da Silva & Meirelles, 2000); and using citrate buffer, PEG and dextran to extract an extracellular lipase from *Burkholderia pseudomallei* (Ooi *et al.*, 2011).

The two-phase aqueous system formed by PEG and arabinogalactan [AG] was first investigated by (Christian *et al.*, 1998) as an alternative to using a PEG-dextran system for separating bovine serum albumin. AG is approximately one fifteenth of the price of dextran (Azevedo*, et al.*, 2009a; Christian*, et al.*, 1998). Unlike maltodextrins and waxy starch from cashew nut tree gum, AG is not susceptible to attack by starch degrading enzymes (Christian*, et al.*, 1998; Sarubbo *et al.*, 2004).

This study investigated the potential of an AG-PEG system to separate whey proteins (α-lactalbumin, β-lactoglobulin and bovine serum albumin (Goff, 2009)). Whey proteins are a mixture of proteins with different biochemical properties. Hence separating whey proteins is similar to separating a biopharmaceutical from a protein mixture (Seader *et al.*, 2011). Unlike many biopharmaceuticals, whey proteins are also inexpensive and relatively easy to obtain

Preliminary attempts were made to optimise system parameters such as pH, salt concentration, initial whey protein dose, and upper to lower phase mass ratio. The former two parameters affect the solubility and the hydrophobicity of the whey proteins (Garrett & Grisham, 2005); the latter two affect the driving force for mass transfer (Albertsson, 1986). All affect separation performance.

Materials and Methods

Larch arabinogalactan powder [AG] (Lonza FiberAid^{TM}) was kindly supplied by Lonza Inc. (Allendale, NJ, USA); polyethylene glycol powder [PEG] (average M_w = 8000) was purchased from Dow Chemical (NZ) Ltd. (Auckland, New Zealand) and whey protein isolate [WPI] powder was purchased from Punch Supplements Ltd (Auckland, New Zealand). Note that the WPI to contain negligible bovine serum albumin (BSA). These powders were used in dry form and mixed with distilled water to prepare standard solutions: AG (40% w/w); PEG (20% w/w); WPI (20 mg/ml; 150 mg/ml).

α-lactalbumin (α-lac) and β-lactoglobulin (β-lac) were purchased from Sigma-Aldrich (Auckland, New Zealand) as lyophilised powders. They were mixed with distilled water to prepare standard protein solutions (5 mg/ml and 10 mg/ml, respectively).

Stock solutions of citrate buffer (100 mM, pH 3.0; 100 mM, pH 5.4; 10 mM + 100 mM KCl, pH 5.4), phosphate buffer (100 mM, pH 7.4), potassium chloride [KCl] (1 M) and sodium hydroxide (1M) were prepared using laboratory dry stock chemicals. 1M NaOH was used to correct the pH of the buffers. Note that the quoted buffer strengths do not account for the minor changes in molarity caused by the pH correction.

The densities of the stock solutions were determined by weighing a fixed volume of each solution.

Turbidimetric titration

The phase equilibrium curve of Christian*, et al.* (1998) was verified by the second turbidimetric titration method of Albertsson (1986). Specified masses of AG and PEG powder were added to a 50 mL centrifuge tube; the system was then made up to a total mass of 25 g with distilled water. The centrifuge tube was then capped and vortex mixed at 2100 rpm for 30 s, creating a turbid suspension. Distilled water was added dropwise from a burette until the system became transparent. The volume of water added was recorded and the mass composition of the system was determined by mass balance.

Tie line verification

Polarimetry and turbidmetry were used to verify the phase-equilibrium tie lines obtained by Christian*, et al.* (1998). In both cases samples of AG-PEG two phase aqueous systems were prepared by adding known masses (based upon the X_1-X_2 tie line of Christian*, et al.* (1998)) of AG and PEG powder to a 50 mL centrifuge tube and adding distilled water to form a 30 g system. These solutions were then vortex mixed for 30 s at 2000 rpm and centrifuged at 2000 ECF for 5 minutes.

Standard solutions of AG were prepared by adding known masses of AG to 15 mL centrifuge tubes and adding distilled water to form a 10 g system. The centrifuge tubes were then capped and vortex mixed at 2000 rpm for 30 s.

All samples and standards were then equilibrated to 30°C in a serological water bath. A polarimeter (Na D lamp, 20-cm tubes) was used to record the optical rotation of the samples and compare them to the standards and specific optical rotation results obtained by Christian*, et al.* (1998). All samples and most standards proved too concentrated for the instrument, and a 100× dilution did not overcome the problem. Hence the polarimetric tie-line verification was abandoned.

The samples and standards were re-equilibrated to 25°C in a serological water bath and turbidity measurements were taken using a Hach 2100P turbidimeter. The standards were used to graphically

correlate turbidity (Nephelometric Turbidity Units [NTU]) and AG concentration (wt %) and the results used to determine the AG content of the samples. The PEG concentration was determined from the phase equilibrium curve of Christian*, et al.* (1998). Note that the contribution of PEG to turbidity was assumed to be negligible.

Standard curves of absorbance against whey protein concentration

Preliminary spectrophotometric scans of α-lac and β-lac solutions (2 mg/ml protein in buffer) failed to find a unique wavelength for each protein, but confirmed that all whey proteins strongly absorb 280 nm light. Absorbance measurements of blank top phases determined that AG strongly absorbed 280 nm light. Hence all further measurements were carried out at 100x dilution.

Simulated 100x dilute upper phases with a total mass of 5 g were created by adding calculated volumes of stock AG solution, PEG solution, WPI solution (20 mg/ml); buffer solution and KCl solution (where appropriate) to a 15 mL centrifuge tube. The centrifuge tubes were then capped and vortex mixed for 30 s at 2000 rpm. The concentrations of whey protein in each phase ranged from 0.0 to 2.0 mg/ml.

A Heλios® UV-Visible spectrophotometer was blanked against samples free of whey protein and used to measure the absorbance of each sample at 280 nm, and the results used to prepare a standard curve of absorbance and whey protein concentration.

Whey Partitioning Experiments

Two-phase aqueous systems with a total mass of 10 g were created by adding calculated volumes of stock AG solution, PEG solution, WPI solution (150 mg/ml); buffer solution and KCl solution (where appropriate) to a 15 mL centrifuge tube (Table 1). The resulting mass fractions of AG and PEG can be found in Table 2. These solutions were then vortex mixed for 30s at 2000 rpm and centrifuged at 2000 ECF for 5 minutes. Each run was performed in triplicate.

Following centrifugation, the volumes of the upper and lower phases were recorded. Samples of the top phase were taken and diluted 100× by volume. The absorbance of each dilute sample was then measured at 280 nm. Protein concentration was determined from the standard curve and the volume ratio was used to determine the partition coefficient (K_p):

$$K_p = \frac{Mass\ of\ protein\ in\ the\ upper\ phase}{Mass\ of\ protein\ in\ the\ lower\ phase}$$

Table 1. Whey partitioning system properties

Run	pH	KCl [mmol/ g solution]	Upper : Lower Phase Mass Ratio	WPI [mg WPI/g solution]
1	3.0	0	1:1	20

2	3.0	100	1:1	20
3	5.4	0	1:1	20
4	5.4	100	1:1	20
5	7.4	0	1:1	20
6	7.4	100	1:1	20
7	5.4	100	1:1	10
8	5.4	100	3:1	20
9	5.4	100	3:1	10

Table 2. Whey partitioning system AG and PEG mass fractions

Upper: Lower Phase Mass Ratio	x(AG) (% w/w)	x(PEG) (% w/w)
1.0	17.80%	7.20%
3.0	9.25%	10.50%

Spectrophotometric arabinogalactan correction

The original whey partitioning method assumed that the PEG-AG phase equilibrium was unaltered by the presence of salts and protein, limiting its accuracy. Preliminary scans indicated that whilst both AG and WPI absorbed 280 nm light, only AG absorbed 320 nm light. Hence standard curves of absorbance (280 nm; 320 nm) against AG concentration, and absorbance (280 nm) against WPI concentration were prepared.

The best two runs (four and seven) from the original partitioning experiments were then repeated at 13% of their original protein concentration, and a blank system (i.e. without WPI) was also prepared. These runs are summarised in Table 3. All runs were performed in triplicate. 320 nm absorbance readings were used to determine the AG concentration in the top phase. Hence 280 nm absorbance measurements were corrected for AG content by deducting an absorbance value determined from standard curve of 280 nm absorbance against AG concentration.

An additional three triplicates of run ten (Table 3) were then tested for upper-phase and lower-phase AG concentration using 320 nm absorbance readings.

Table 3. Whey partioning systems verified by spectrophotometric methods

Run	Original Run	pH	KCl [mmol/ g solution]	Upper : Lower Phase Mass Ratio	WPI [mg WPI/g solution]
10	–	5.4	100	1:1	0
11	7	5.4	100	1:1	1.3
12	4	5.4	100	1:1	2.7

Fast protein liquid chromatography (FPLC)

An upper phase sample (10x dilute) from run four of the whey partitioning experiments was run on an Äkta FPLC fitted with a Superdex 200 10/300 column in citrate buffer (10mM + 100 mM KCl, pH 5.4). The sample volume was 50 µL and the flowrate was 0.5 ml/min. Samples of WPI (20 mg/ml), α-lac (5 mg/ml); β-lac (10 mg/ml); AG (8% w/w) and PEG (20% w/w) were run under the same conditions. The mass ratio of α-lac to β-lac in the WPI was calculated using extinction coefficients of 28540 $M^{-1}cm^{-1}$ and 17600 $M^{-1}cm^{-1}$, respectively (Engel, van Mierlo and Visser, 2002; Collini, D'Alfonso and Baldini, 2000). The molar masses used in the calculations were 14000 $gmol^{-1}$ and 18400 $gmol^{-1}$, respectively (Sigma-Aldrich, 2014).

Results and Discussion

Turbidimetric Titration

The results of the turbidimetric titration are summarised in Table 4. Note that each set contained three triplicates. The results for sets one and three agree with the published results of Christian*, et al.* (1998), but the results from set two indicate a lower than expected PEG concentration. This was probably due to the difficulty of determining the end point of the turbidimetric titration. Solutions rich in AG are a dark brown colour, and this makes it difficult to determine the precise point at which the turbid solution becomes clear. Future titrations should use the first turbidimetric titration method of Albertsson (1986), which adds PEG solution of a known concentration to a fixed volume of AG solution of known concentration until turbidity ensues.

Table 4. Turbidimetric titration results

Set	x_a(AG) (% w/w)	x_a(PEG) (% w/w)	x_a(H_2O) (% w/w)	Relative Uncertainty
1	14.94	5.52	79.53	0.35%
2	18.06	1.92	80.03	4.27%
3	5.24	9.02	85.74	0.79%

$$Relative\ Uncertainty = \frac{Standard\ Deviation}{\sqrt{\#\ Replicates} \times Mean}$$

Tie Line Verification

The correlation between turbidity and arabinogalactan concentration (Figure 1) is approximated by a straight line when the solution contains less than 20% AG by mass. Above this concentration the curve approaches an asymptote of approximately 100 NTU.

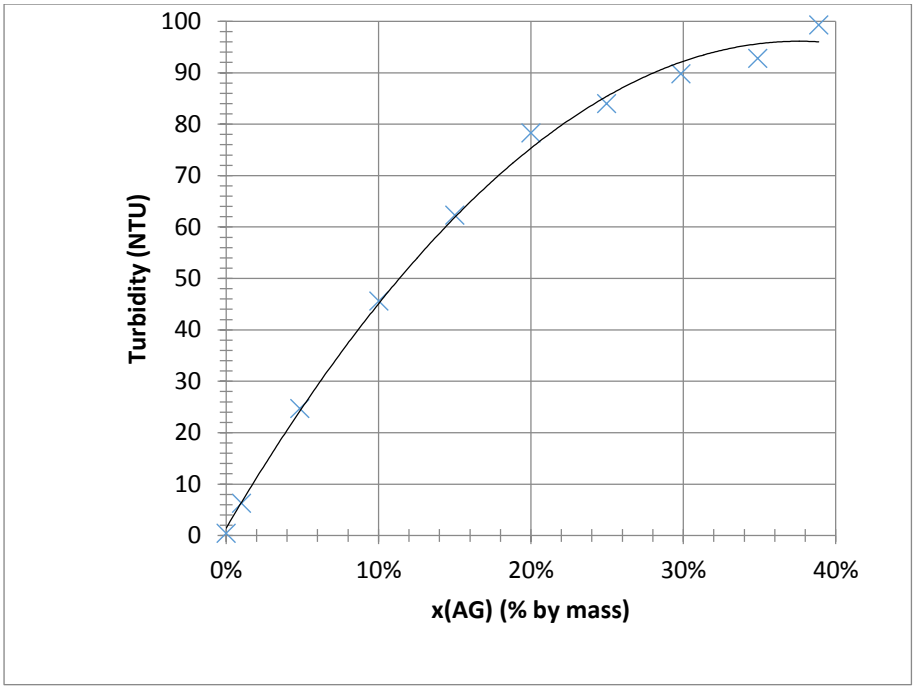

Figure 1. Initial standard curve of turbidity against AG concentration (% w/w)

The turbidity standards deteriorated rapidly with age, making it necessary to prepare fresh turbidity standards every day, although this was only done for AG concentrations of up to 10% by mass. The final plot used to verify the tie lines is shown in Figure 2.

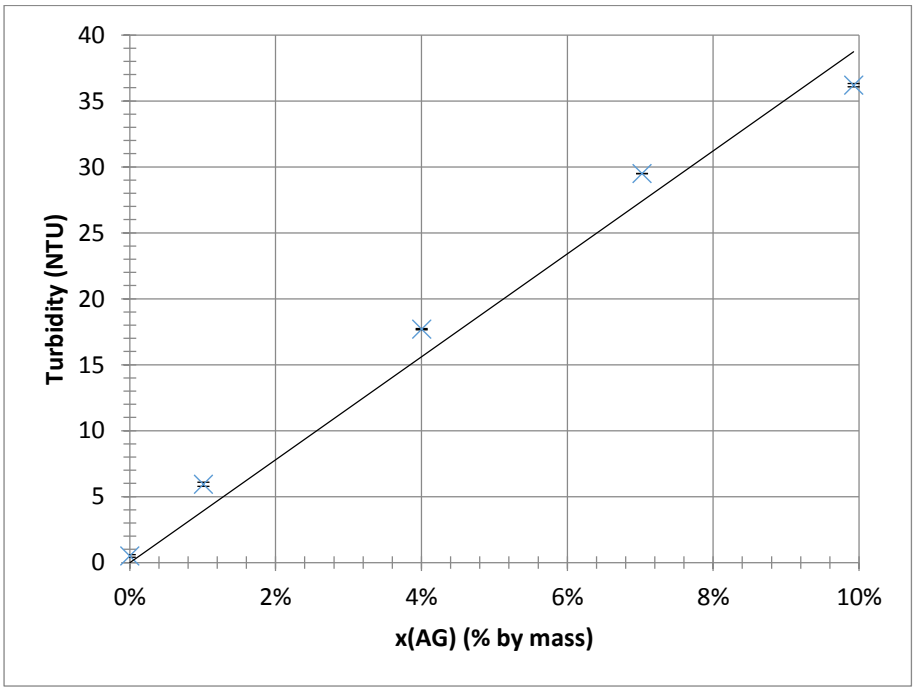

Figure 2. Final standard curve of turbidity against AG concentration (% w/w)

In most cases, AG concentration of the top phases was found to be between 0.4% and 0.45% by mass, which is within the expected range (i.e. less than 1% AG w/w), but inconsistent with the predicted result (approximately 0.72% w/w). As the gradient of the curve was steep, this variation

could be easily explained by the error of the instrument (1 NTU). Using 280 nm absorbance measurements may be a more reliable method of determining AG concentration.

Standard Curves of Absorbance and Whey Protein Concentration

The calibration curves of absorbance and whey protein concentration (Figure 3) indicate that the spectrophotometer is best used for WPI concentrations less than 1 mg/ml, as the blank top phase absorbed approximately 25% of the 280 nm UV light emitted by the detector.

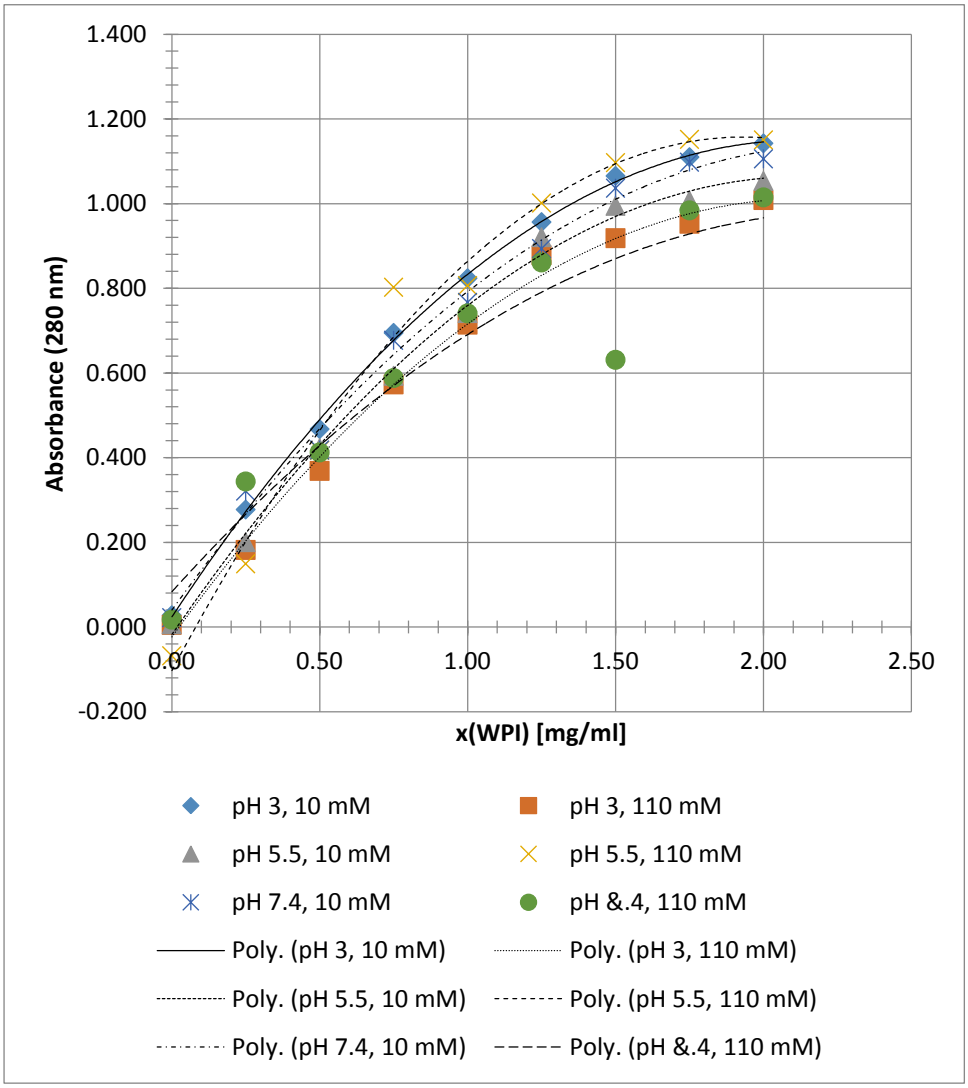

Figure 3: Standard curves of 280 nm absorbance against WPI concentration.

Whey Partitioning Experiments

The top phases of systems 1, 2, 3, 5 and 6 had negligible absorbances, indicating all the WPI was still in the lower phase. System four and its optimisation, system seven had substantial absorbances in the top phase (>0.1), indicating that substantial protein separation had taken place (Table 5, Table 6 Table 7).

System four exhibited a strong, but realistic recovery of WPI. It had an overall WPI partition coefficient of 1.50 and 60.0% recovery of WPI in the upper phase. This suggests that the WPI has a stronger affinity for the upper phase. Hence this system could potentially be used for commercial scale extraction of whey proteins or similar biological compounds.

The results from system eight indicate that increasing the relative mass of the top phase greatly decreases protein recovery. However the effect of decreasing the relative mass of the upper phase is unknown. Hence the system containing: 17.20% (w/w) AG; 7.20% (w/w) PEG; 10 mmol/g citrate buffer (pH 5.4); 100.0 mmol/g KCl; and 20 mg/g of WPI worked well, but little optimisation has been carried out in this region. Future work should attempt to optimise these parameters, using the values used in this work as a starting point.

The data from system seven is less conclusive, but suggest protein recovery can be enhanced by lowering the initial WPI dose. This is consistent with WPI precipitate being observed in the bottom phase of most AG-PEG systems. However the theoretical WPI recovery for this system exceeds 100%, which is not realistic.

Table 5. Absorbance measurements and upper phase protein concentrations for high-performing AG-PEG two-phase aqueous extraction systems

Run	Absorbance	Dilution Factor	Upper Phase Protein Concentration (mg/mL)
4	0.220	0.01	22.6
7	0.318	0.01	35.5

Table 6. Volume ratios for high performing AG-PEG two-phase aqueous extraction systems

Run	Upper Phase Volume (mL)	Lower Phase Volume (mL)	Total System Volume (mL)
4	5.33	3.67	9.00
7	5.30	3.37	8.67

Table 7. Protein recovery parameters for high performing AG-PEG two-phase aqueous-extraction systems

Run	Total WPI in System (mg)	WPI in Upper Phase (mg)	Upper Phase WPI Recovery (% w/w)	Overall WPI Partition Coefficient (K_p)
4	201.0	121	60.0%	1.50
7	101.4	188	185.5%	-2.17

The most likely cause of these high absorbance readings was the blank used to zero the spectrophotometer. All blanks in the experiments were simulated top phases made up with AG, PEG and distilled water to match the compositions found on the phase equilibrium diagram of Christian, *et al.* (1998). As AG absorbs 280 nm ultraviolet light, even a slight error in the phase composition could cause the protein concentration to be overestimated. This problem could potentially be overcome by spectrophotometric correction.

In general, all successful WPI separations were carried out at a pH 5.4, in the presence of 10 mmol/g KCl. Separations carried out at pH 3.0 or 7.4 or in the absence of KCl were unsuccessful, indicating that separation success is closely related to pH and salt concentration.

The pH optimum could be explained by pH 5.4 being the experimental pH closest to the isoelectric point of whey proteins (5.0) (Goff, 2009). As the PEG-rich upper phase is more hydrophobic (Christian*, et al.*, 1998), having a system pH close to the isoelectric point is likely to make the whey proteins more soluble in hydrophobic solutions by reducing the ionisation of amino acids (Garrett & Grisham, 2005).

The presence of 100 mmol/g of KCl in the most effective separation systems is unremarkable, as it is well known that low concentrations of salt can enhance the solubility of proteins (Garrett & Grisham, 2005). However as KCl breaks down water structure (i.e. is a chaotropic salt), it favours protein migration to the hydrophobic phase (Rosa*, et al.*, 2010). It would be interesting to see how the use of a water structure-stabilising (i.e. kosomtropic) salt affects protein separation performance.

Spectrophotometric arabinogalactan correction

The standard curves for AG (Figure 4) and WPI (x-r) indicate a linear correlation between absorbance and concentration of AG and WPI. Hence 320 nm absorbance measurements can be used to correct 280 nm absorbance measurements for AG absorbance, as AG does not absorb 320 nm light.

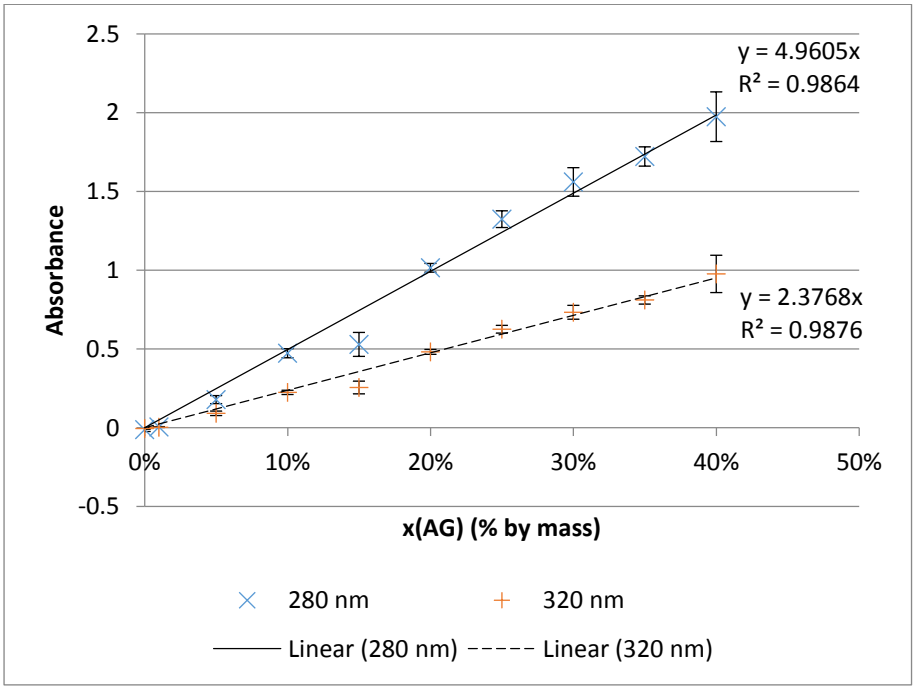

Figure 4. Standard curve of absorbance at 280 nm and 320 nm against AG concentration (% w/w)

The results of the partitioning experiments (Table 8 – Table 10) were inconclusive. The low initial concentration of WPI meant that the whey protein that migrated into the upper phase was barely detectable, and hence there was a large measurement error. The upper phase AG concentration was

also much higher than expected (< 1 wt%), and decreased in the presence of WPI. Similarly, the additional triplicates of run ten had upper-phase and lower-phase AG concentrations of 15.4 wt% and 13.8 wt%, respectively, which contradicts the phase equilibrium-predictions of Christian, *et al.* (1998). Hence further work is needed to accurately determine the concentration of AG and WPI in a PEG-AG extractive system.

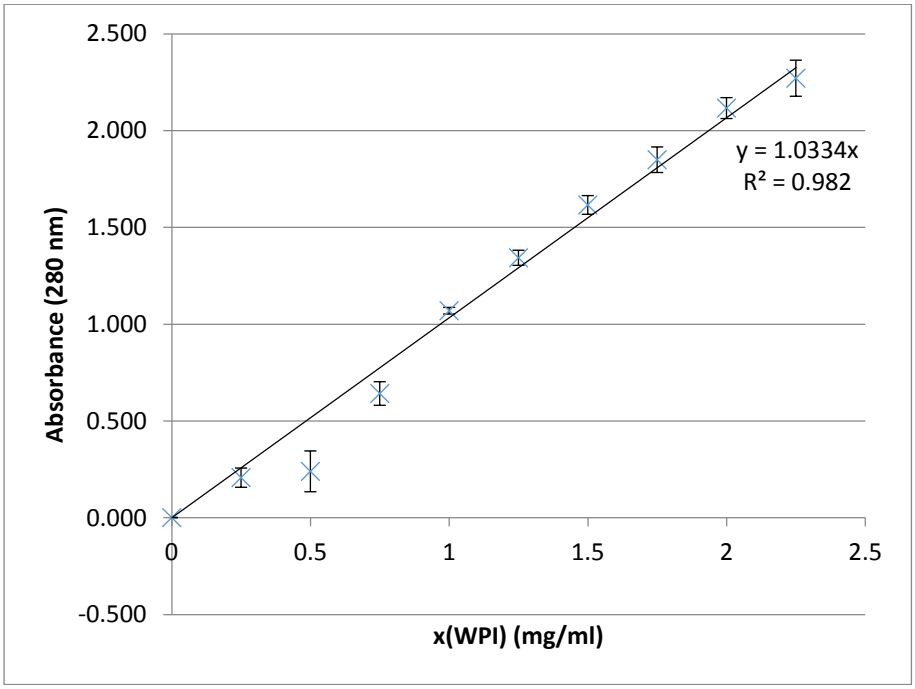

Figure 5. Standard curve of absorbance at 280 nm against WPI concentration (mg/ml)

Table 8. Absorbance measurements and upper phase arabinogalactan and protein concentrations for AG-PEG two-phase aqueous extraction systems at a reduced protein dose.

Run	Absorbance (280 nm)	Absorbance (320 nm)	Dilution Factor	Upper Phase AG Concentration (wt %)	Upper Phase Protein Concentration (mg/ml)
10	1.044	0.491	0.01	20.7(3)	0.02(2)
11	0.940	0.430	0.01	18.1(5)	0.04(4)
12	0.939	0.433	0.01	18.2(4)	0.04(3)

Table 9. Volume ratios for high performing AG-PEG two-phase aqueous extraction systems at a reduced protein dose.

Run	Upper Phase Volume (mL)	Lower Phase Volume (mL)	Total System Volume (mL)
10	6.0	3.5	9.5
11	5.8	3.5	9.3
12	5.9	3.5	9.4

Table 10. Protein recovery parameters for high performing AG-PEG two-phase aqueous-extraction systems at a reduced protein dose.

Run	Total WPI in System (mg)	WPI in Upper Phase (mg)	Upper Phase WPI Recovery (% w/w)	Overall WPI Partition Coefficient (K_p)
10	0.000	0.11(13)	–	–
11	26.860	0.26(25)	1.0%	0.01

12	13.554	0.21(18)	1.6%	0.02

Fast Protein Liquid Chromatography (FPLC)

Stacked traces from the FPLC run are shown in Figure 6. A comparison of peaks reveals that α-lactalbumin (α-lac) is the main protein extracted into the top phase. A small amount of β-lactoglobulin (β-lac) is present in the sample, but at a much lower concentration than α-lac. This is an interesting result, as the FPLC data indicates that the mass ratio of α-lac to β-lac is ca. 8:25, which is consistent with Goff (2009). Hence the α-lac has been preferentially extracted into the upper phase, although the precise extraction mechanism remains unknown.

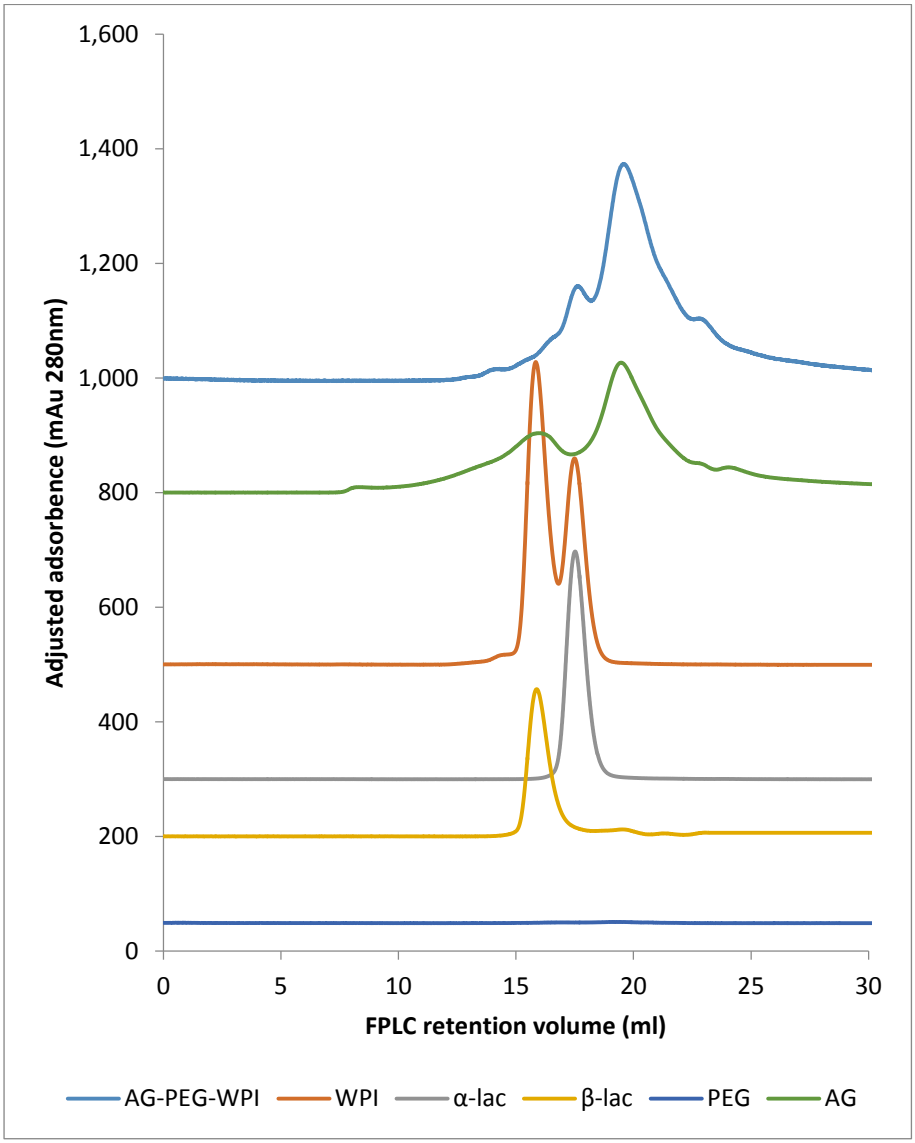

Figure 6. Stacked FPLC traces for various samples in 10 mM pH 5.4 citrate buffer + 100 mM KCl

The traces also show that AG is responsible for blank samples absorbing 280 nm UV light. PEG does not respond to 280 nm light, as its trace does not contain any peaks.

Although no definitive quantification was carried out, the AG-PEG-WPI sample appears to contain much more AG than α-lac. The ease of separating AG from the α-lac will be an important consideration for future studies and the economics of any commercial applications that arise from these results.

Conclusions

A two phase aqueous system containing 17.20% (w/w) arabinogalactan (AG); 7.20% (w/w) polyethylene glycol (PEG); 10 mmol/g citrate buffer (pH 5.4); 100.0 mmol/g KCl; and 20 mg/g of whey protein isolate (WPI) extracted 60% (w/w) of the WPI to the top phase. The protein extract was much richer in α-lactalbumin than the original WPI, indicating that preferential partitioning of the whey proteins took place. This makes AG-PEG two-phase aqueous extraction a potentially useful method for reducing the use of packed bed chromatography in the downstream processing of biopharmaceutical products. However the relatively low concentration of whey protein relative to AG means that packed bed chromatography will still be required to separate the two compounds.

Reducing the dose of WPI from 20 mg/g to 10 mg/g improved protein recovery, but the theoretical protein recovery exceeded 100%, which is unrealistic. This was likely due to the blank used to zero the spectrophotometer. Absorbance correlates linearly with concentration of AG (280 nm and 320 nm), and WPI (280 nm). However attempts to use a 320 nm to 280 nm absorbance ratio to correct 280 nm absorbance measurements for AG content yielded unrealistic results. Additionally, the arabinogalactan content in the upper phase decreased in the presence of protein. Hence the 60% (w/w) protein recovery estimate may be conservative.

Increasing the relative mass of the upper phase appeared to greatly reduce the efficacy of protein separation, but the effect of decreasing it remains unknown. Future studies should optimise the pH, citrate buffer concentration and KCl concentration using pH 5.4, 10 mmol/g citrate buffer and 100 mmol/g KCl as a starting point. Future studies should also investigate the discrepancy between predicted and actual AG concentration in the upper and lower phases. The effect of chaotropic and kosomtropic salts on separation performance should also be investigated.

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Biography

Timothy W.C. Ng

Timothy is a Doctoral Fellow at the University of Waikato, and is currently working toward a Doctor of Philosophy (PhD) degree. His working thesis title is *The production of a protein-rich and lipid-rich aquaculture feed from green wastes.* Timothy holds a Bachelor of Engineering (Honours) in Chemical and Biological Engineering from the University of Waikato. He has also been the President of the University of Waikato chapter of the Golden Key International Honour Society since October 2012. He is also an Associate Member of the Institution of Chemical Engineers and a Graduate Member of the Institution of Professional Engineers of New Zealand.

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