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ABSTRACT

Whey protein isolate (WPI) solutions (100 g L^{-1} protein) were subjected to a heat-treatment of 80 ºC for 10 min. Un-heated and heat-treated WPI solutions were hydrolysed with Corolase® PP at pilot-scale to either 5 or 10 % degree of hydrolysis (DH). Hydrolysates were subsequently processed via cascade membrane fractionation using 0.14 µm, 30, 10, 5 and 1 kDa molecular weight cut-off membranes. The compositional and molecular mass distribution profiles of the substrate hydrolysates and membrane processed fractions were determined. Whole and fractionated hydrolysates were assayed for both angiotensin-I-converting enzyme (ACE) inhibitory activity and ferrous chelating capabilities. A strong positive correlation ($P < 0.01$) was established between the average molecular weight of the test samples and the concentration needed to chelate 50 % of the iron (CC_{50}) in solution. The lowest ACE inhibition concentration (IC_{50} = 0.23 g L^{-1} protein) was determined for the 1 kDa permeate of the heat-treated hydrolysate hydrolysed to 10 %DH.

KEYWORDS: hydrolysis; ACE; iron-binding; pilot-scale; bio-functional hydrolysates; heat-treatment

1. Introduction

The functional properties of proteins are related to their structural characteristics. Processing steps, such as heat-treatment, can induce structural alterations in whey proteins which may affect protein techno- and bio-functional attributes. These alterations modify the physico-chemical characteristics (O'Loughlin, Murray, Kelly, FitzGerald, & Brodkorb, 2012) and susceptibility to enzymatic hydrolysis (Boye, Ma, & Harwalker, 1997). The enzymatic release of bio-functional peptides from whey proteins has resulted in the generation of ingredients with potential to, for example, control hypertension (Tavares, Contreras, Amorim, Pintado, Recio, & Malcata, 2011) and enhance mineral binding (Kim, Seo, Khan, Ki, Nam, & Kim, 2007b).

Hypertension induces blood vessel constriction and a reduction in elasticity as well as hypertrophy and collagen deposition within the vascular system resulting in increased risk of cardiovascular diseases (Dézsi, 2000). Upon renin cleavage of angiotensinogen to release angiotensin I, the glycoprotein angiotensin-I-converting enzyme (ACE) converts angiotensin I to the potent vasoconstrictor angiotensin II. Much research has been conducted into bio-functional ingredients which possess ACE-inhibitory activity for incorporation into food products to aid, or reduce the reliance on, antihypertensive drugs, such as Captopril®. Numerous reports of whey protein derived inhibitory peptides exist in the literature (Morris & FitzGerald, 2008; Mullally, Meisel, & FitzGerald, 1997; Norris & FitzGerald, 2013).

Iron deficiency affects an estimated 2 billion people worldwide (Zimmermann & Hurrell, 2007). The inter-conversion of iron to its different oxidation states is utilised by biological systems for electron transfer, ligand binding, cell growth and differentiation. Iron needs to be in a soluble format in order to be effectively

absorbed. Direct fortification of food products with iron is problematic due to its reactivity and ability to generate reactive oxygen species (ROS) and low solubility (Fidler et al., 2004). Thus, interest in mineral-binding hydrolysates has focused on their ability to reduce the reactivity and enhance the solubility of iron in fortified foods (Ait-Oukhatar et al., 2002).

For these reasons iron-binding hydrolysates and fractions derived from whey protein substrates have garnered significant interest of late. Iron-binding peptide complexes have been produced through hydrolysis, e.g., alcalase hydrolysis of β-lactoglobulin (Zhou et al., 2012). From this study an optimum mass ratio for complex formation at neutral pH was determined to be 40 (hydrolysate): 1 (iron, as $FeCl_3$). Peptic hydrolysis of lactoferrin can liberate 'lactoferricin', *f*(17-41), which has been shown to exhibit iron-binding capacity (Vegarud, Langsrud, & Svenning, 2000; Wakabayashi, Takase, & Tomita, 2003). Fractionated hydrolysates of heated (100 ºC, 10 min) WPC high in alanine, phenylalanine and lysine with improved iron-binding capabilities have been reported (Kim et al., 2007a). Also, digestion of α-la-enriched formulas have exhibited increased absorption of iron using infant rhesus monkeys as a animal model (Kelleher, Chatterton, Nielsen, & Lonnerdal, 2003). Hydrolysates may be subsequently formulated into products demonstrating enhanced mineral absorption characteristics, similar to, for example Capolac® (Arla Food Ingredients).

While recent investigations into dual functionalities and thermal pre-treatments of whey proteins have been published (Adjonu, Doran, Torley, & Agboola, 2013), there remains little literature available on the kinetics of pilot / semi-pilot scale WPI hydrolysis and the compositional and bioactive properties of, and consequences for, resulting hydrolysates. The primary objectives of this study were two-fold. Firstly, the scale-up of a high viscosity, aggregated WPI dispersion with limited

solubility from a previous thermally-denatured WPI laboratory hydrolysis process (O'Loughlin et al., 2012). Secondly, investigation of the bio-functional, namely; anti-hypertensive and ferrous chelation, attributes of these hydrolysates and membrane processed fractions of heat-treated and un-heated control WPI dispersions. Concomitant relationships between selective process treatments and bio-functionalities were also investigated.

2. Materials and methods

2.1 Materials

Whey Protein Isolate (Isolac®) was provided by Carbery Food Ingredients, (Ballineen, Co. Cork, Ireland). The powder contained 89.3 % (w/w) protein by Kjeldahl [*N* x 6.38, (Merrill & Watt, 1973)]. The level of denaturation of the starting material was 6.8 % w/w determined via isoelectric precipitation at pH 4.6 (Parris & Baginski, 1991). The digestive-enzyme complex Corolase® PP was from AB Enzymes GmbH (Darmstadt, Germany) and is compositionally described elsewhere (Mullally, O'Callaghan, FitzGerald, Donnelly, & Dalton, 1994). Corolase® PP possesses GRAS status and high proteolytic activity in whey producing non-bitter hydrolysates (Svenning, Brynhildsvold, Molland, Langsrud, & Vegarud, 2000).

All other reagents and chemicals were procured from Sigma-Aldrich (Dublin, Ireland) unless otherwise stated. All experiments were determined in duplicate unless otherwise stated.

2.2 Pilot-scale heat treatment and enzymatic hydrolysis of WPI

WPI was reconstituted to 107 g L^{-1} on a protein basis with reverse-osmosis water (RO) in a jacketed 316-grade stainless steel vessel fitted with a gated scrape

surface agitator. The WPI dispersions (at pH 6.4) were adjusted to pH 8.0 with 4 N NaOH and then diluted to 100 g L^{-1} protein with RO water to a final volume of 400 L. Heat-treated WPI dispersions were brought to 80 °C for 10 min by indirect steam injection before cooling to 50 ºC utilising chilled water.

Enzymatic hydrolysis at 50 ºC was initiated by the addition of Corolase® PP. The pH was maintained at pH 8.0 throughout the reaction by manual titration with 4 N NaOH using a Sandpiper® air-operated double-diaphragm polypropylene pump (Warren Rupp Inc., OH, USA) and a DEM JCS-60 floor scales (DEM Machines Ltd., Naas, Ireland) to monitor base addition. The degree of hydrolysis (DH) was determined from the volume of base consumed (Adler-Nissen, 1986; O'Loughlin et al., 2012). The enzyme:substrate ratio (E:S) employed varied depending on the target DH. An E:S of 2:300 (0.67 %, w/w) on a protein equivalent basis was utilised to generate hydrolysates at a DH of 5 %, whereas for 10 %DH hydrolysates an E:S of 1:100 (1.0 %, w/w) was employed. The reaction was terminated by heating the hydrolysate solutions 85 ºC for 25 sec in a Unison H17 109 plate-and-frame 53 L heat-exchanger plant (Unison Engineering Services Ltd., Limerick, Ireland). A 50 L sample of the heat-inactivated hydrolysate was withdrawn, evaporated (to ~40 % total solids) on a Tetra Scheffers™ falling-film single-stage evaporator (Tetra Pak, Gorredijk, The Netherlands) and spray dried using a pilot scale Anhydro Lab 3 spray drier (SPX Flow Technology A/S, Soeborg, Denmark). Inlet and outlet temperatures during spray drying ranged from 185 – 190 and 85 – 90 °C, repectively.

2.3 Microfiltration and ultrafiltration of WPI hydrolysates

The remaining hydrolysate sample (~350 L) was subjected to cascade membrane fractionation utilising a GEA Model F unit (GEA Process Engineering

A/S, Skanderborg, Denmark). For hydrolysates at 5 % and 10 % DH which were derived from heat-treated WPI, intial fractionation was via microfiltration (MF). MF was accomplished with three Tami Isoflux™ ceramic membranes (25 x 1178 mm, 23 channels, Tami Industries, Nyons Cedex, France) having a nominal molecular weight cut off (MWCO) of 0.14 µm and a total membrane area of 1.05 m^2. MF was carried out at 50 ºC to a volume concentration factor (VCF) of 8. A feed recirculation rate of 1500 L h^{-1} at 1 bar and membrane inlet pressure of 4.2 bar were maintained throughout processing. For hydrolysates to 5 %DH of un-heated control substrates two 30 kDa MWCO Koch KMS HFK™-328 spiral wound membranes (96 x 965 mm, Koch Membrane Systems, Wilmington, MA, US) were used as the initial fractionation step. For control hydrolysates to the 10 % DH level two 10 kDa MWCO Koch spiral wound membranes were utilised.

In all cases, the subsequent permeate stream (~ 300 L) prepared above was then subjected to ultrafiltration (UF) using the same GEA model F unit fitted with two Koch KMS HFK™-328 spiral wound membranes. These membranes have a nominal MWCO of 5 kDa and a total surface area (TMA) of 11.2 m^2. The 5 kDa permeate stream (~300 L) was then processed (at 50 ºC) on the GEA model F plant fitted with two 1 kDa MWCO Alpha Laval UF-ETNA spiral wound membranes (TMA 11.2 m^2, 95 x 965 mm, Alpha Laval AB, Lund, Sweden) to a VCF of 3, whereupon feed volume was returned to 300 L with RO and UF was carried out to a final VCF of 7. A feed recirculation rate of 1500 L h^{-1} at 1 bar and membrane inlet pressure of 5 bar were maintained throughout processing. All process streams, with the exception of 1 kDa retentates and permeates, were dehydrated in a pilot scale Anhydro Lab 3 spray drier using the conditions as described above. The 1 kDa retentates and permeates were further concentrated (to ~35 % total solids) before spray drying, as outlined

above, in a Anhydro F1 Lab single effect falling film evaporator. As an example, the process flow diagram for the production of fractionated powders from hydrolysed (to 10 % DH) heat-treated WPI is shown in Appendix A (Fig. A. 1). Final volumes prior to concentration and total solids levels are provided in Appendix B for all four processes (Fig. B. 1).

2.4 Particle size, chromatography and compositional analysis of control and heated-treated WPI and hydrolysates

Particle size analysis was carried out using a Malvern Mastersizer MSS (Malvern Instruments Ltd., Worcestershire, U.K.) according to the protocol of O'Loughlin et al. (2012). Size-exclusion chromatography (SEC) on samples was carried out according to the protocol of O'Loughlin et al. (2013b).

Protein content was determined by micro-Kjeldahl on a Foss Kjeltec™ 8400 (Foss, Hillerød, Denmark). The procedure was modified from Koops et al. 1975 (Koops, Klomp, & Elgersma, 1975) where a conversion factor of 6.38 was used in accordance with Merrill, 1973 (Merrill et al., 1973). Ash was determined gravimetrically through modification of the International Dairy Federation (IDF) method (International Dairy Federation, 2008) where ≥ 1 g of powder was weighed to the nearest 0.1 mg. Moisture content was determined through heating at 102 °C ± 2 °C for 2 hrs according to the respective IDF method (International Dairy Federation, 2004). Lactose was determined using a food analysis lactose/galactose test kit (Roche Diagnostics GmbH, Mannheim, Germany). Determination of free-amine (NH_2) groups was determined via the 2,4,6-trinitrobenzene 1-sulfonic acid (TNBS) methodology of Adler-Nissen (1979) modified by O'Loughlin et al. (2012).

2.5 Angiotensin-I-coverting enzyme inhibition assay

ACE was extracted from rabbit lung acetone powder reconstituted in 100 mM sodium borate buffer according to the method of Cushman & Cheung (1971). Subsequently, the inhibition of ACE activity was measured by HPLC through modification of the method of Jiang et al. (Jiang, Tian, Brodkorb, & Huo, 2010). Hippuryl-histine-leucine (Hipp-his-leu), 1mM, was dissolved in 50 mM sodium borate buffer (pH 8.3) containing 0.5 M NaCl. A mixture containing 120 μL Hipp-his-leu solution and 20 μL sample (10 g L^{-1}) was pre-incubated at 37 ºC for 5 min whereupon 10 μL of ACE solution (~ 133 nano-katal at 37 ºC) was added and the mixture incubated for 60 min. The reaction was stopped by the addition of 150 μL of 1 M HCl. Hippuric acid liberated by ACE was determined by RP-HPLC where samples were eluted under gradient elution using a Symmetry C18 (2.1 mm x 150 mm, 5 μm) from Waters (Milford, MA, USA) at 28 ºC. Samples were filtered through 0.45 μm filters (Sartorius Stedim Biotech GmbH, Germany) and 5 μL was injected onto the column. The gradient elution conditions were as follows; 0 to 100 % solvent B for 20 min, 100 % solvent B for 5 min, 100 to 0 % solvent B for 5 min and 0 % solvent B for 5 min at a flowrate of 0.2 mL min^{-1}. Solvent A was Milli-Q water, 0.1 % TFA (v/v) and solvent B was 100 % MeCN, 0.1 % TFA (v/v). The absorbance of the eluent was measured at 228 nm. A response curve for hippuric acid ranging from 0.5 – 5 mM gave the following equation of the line;

$$y = 4.0 \times 10^{6}x + 461732 \ (R^2 = 0.995) \qquad \text{(Eqn. 1)}$$

The percentage ACE inhibitory activity was calculated according to the following formula;

ACE inhibitory activity (%) = $[(A_0 – A_1) / A_0]\cdot 100$ (Eqn. 2)

Where: A_0 was the absorbance at 228 nm of hippuric acid without addition of sample and A_1 was the absorbance of hippuric acid after incubation with sample.

The concentration of selected hydrolysates needed to inhibit the ACE by 50 % (IC_{50}) was determined by assaying % ACE inhibition at different concentrations of hydrolysates. Experiments were conducted in triplicate. Captopril® (*2S*-1-[*2S*-2-methyl-3-sulfanylpropanoyl] pyrrolidine-2-carboxylic acid) was used as a standard where an IC_{50} of 15.4 nM was obtained under assaying conditions.

2.6 Total iron concentration and ferrous chelating activity determination

The concentration of total iron present in the WPI, the hydrolysates and their respective fractions was determined using a modification of the method of Viollier et al. (Viollier, Inglett, Hunter, Roychoudhury, & Van Cappellen, 2000). A standard curve was prepared with 0 – 1,500 μg Fe^{2+} L^{-1} from a standard solution of 1.79 M $FeSO_4.7H_2O$ in 0.5 M HCl. Test samples (1 g L^{-1} protein) were dissolved in 0.5 M HCl and stored overnight. Test samples (200 μL) were added to 1,500 μL of distilled water to which 300 μL of 1.4 M hydroxylamine hydrochloride in 2 M HCl was added. The mixture was vortexed on a IKA® MS2 Minishaker (IKA® GmbH & Co. KG, Staufen, Germany) at 1,000 min^{-1} and allowed to stand at room temperature for 30 min. Following this, 200 μL of ferrozine reagent (0.01 M in 0.1 M CH_3COONH_4) and 300 μL of 5 M CH_3COONH_4 was added and the mixture was allowed to stand for 3 min. The absorbance was read at 562 nm on a Varian Cary® 1 dual beam UV-visible

spectrophotometer (Varian Ltd., Walton-on-Thames, UK). A reaction mixture where the test sample volume was replaced with dH_2O was used as a blank. A response curve for 0 – 1.5 x 10^{-3} g L^{-1} iron (as $FeSO_4.7H_2O$) gave a linear response within this range and the following equation of the line;

$y = 0.0004x + 0.0259$ ($R^2 = 0.999$) (Eqn. 3)

Total iron was then determined according to the following formula;

Total iron ($\mu g\ L^{-1}$) = $[(A_{562} – 0.0259) / 0.0004]$ (Eqn. 4)

The ferrous chelating activity was determined using a modification of the method of Decker & Welch (1990). Test samples (1 mL of 1 g L^{-1}) were mixed with 1,350 μL of dH_2O and 50 μL of 2 mM $FeCl_2$ and the mixture vortexed at 1,000 min^{-1} for 1 min. The reaction was initiated by the addition of 100 μL of 5 mM ferrozine and the samples vortexed again and allowed to stand at room temperature for 10 min. The absorbance was read at 562 nm as described previously. EDTA-Na_2 was used as a positive control for chelation. The ferrous chelating activity was then determined according to the following formula;

Ferrous chelating activity (%) = $[(A_0 – (A_1 – A_2)]/A_0 \cdot 100$ (Eqn. 5)

Where: A_0 was the absorbance of the control at 562 nm, A_1 was the absorbance of the sample at 562 nm and A_2 was the absorbance of the blank at 562 nm.

The concentration of selected hydrolysates needed to chelate 50 % of the ferrous iron (CC_{50}) from 2 mM $FeCl_2$ was determined through assaying 0.5 – 10 g L^{-1} test samples. These chelation experiments were determined in triplicate.

2.7 Statistical analysis

Statistical analysis was performed using Minitab 15 software (Minitab Inc., State College, PA, USA). Data is presented as mean ± SD. Statistical difference of means was determined by one-way ANOVA followed by a Tukey test. For correlation analysis the critical Pearson's r coefficient for $P < 0.01$ was determined to be 0.549.

3. Results and discussion

Background studies by the authors and others have highlighted the significance of using thermally-denatured WPI as a substrate in terms of structural and molecular implications. O'Loughlin et al. (2012) demonstrated the changes arising during Corolase® PP catalysed hydrolysis reaction as a result of heat-induced protein aggregation due to pre-hydrolysis heat-treatments and the consequences of this from a molecular perspective were subsequently studied (O'Loughlin, Murray, Brodkorb, FitzGerald, & Kelly, 2013a; O'Loughlin et al., 2013b).

3.1 Characterisation of heat-treatment, hydrolysis and fractionation of WPI solutions

Heat-treatment (80 ºC, 10 min) of WPI resulted in aggregate formation and a reduction in determined 'native-like' protein (Tolkach, Steinle, & Kulozik, 2005) of ~64 % (results not shown). Using laser light scattering, the particle size was seen to increase from 16.1 ± 0.5 μm (D.v 09) in the control WPI to 58.3 ± 1.1 μm in the heat-

treated dispersion – slightly higher than that previously generated (mean particle size 40.2 ± 0.6 μm) during laboratory scale heat-treatment of WPI (O'Loughlin et al., 2012). The development of smaller particle sizes at laboratory scale was attributed to more efficient agitation using a three-bladed impeller. Pre-heat treatment of WPI dispersions resulted in 29 and 34 % reductions in the time required to reach 5 and 10 % DH, by comparison with the respective hydrolysates from the un-heated control WPI (Fig. 1). These results are in general agreement with those obtained previously at the laboratory scale (O'Loughlin et al., 2012). However, the extent of decrease in reaction time was lower for the pre-heat treated WPI hydrolysates generated at pilot-scale. This may be related to the scale of the experiment (400 L) and the need to employ a lower E:S in order to control the reaction and facilitate efficient heat-inactivation of this larger volume. Also, at pilot scale the particle size difference may be due to altered substrate accessibility.

Heat-treatment of WPI induced a shift in the molecular weight (M_w) distribution of the starting material (Tables 1 and 2). Hydrolysis to 5 %DH of the heat-treated WPI (Table 1) resulted in a higher percentage soluble material < 1 kDa (~34 %) compared to the equivalent control hydrolysate (~27 %). Hydrolysis of the heat-treated WPI resulted in an increase in the percentage soluble material to ~57 % (results not shown). There was a significant ($P < 0.05$) increase in the ash content in the 5 % DH hydrolysate of the heat-treated WPI (~9 %) compared to the control hydrolysate (~7 %). It was not possible to utilize a 30 kDa molecular weight cut-off (MWCO) membrane in the fractionation of the 5 % and 10 %DH hydrolysates of heat-treated (80 ºC for 10 min) WPI due to the high level of insoluble aggregated material (39 ± 2.1 % (w/w), results not shown). Therefore, a 0.14 μm CO MF step was first performed on the hydrolysates obtained from heat-treated WPI resulting in

retentate powders which had a relatively low protein concentration, a high percentage of soluble material > 30 kDa and a high ash content (Table 1). Interestingly, the percentage soluble material 10 – 5 kDa increased in the unhydrolysed substrate, from 3.8 ± 0.9 to 26.1 ± 0.9 %, upon heat-treatment. This was due to the increased insolubility and presence of large aggregates upon heating which resulted in only the soluble fraction being available for distribution analysis.

As expected the higher level of hydrolysis at 10 % DH of unheated control WPI favored an increased percentage soluble material < 1 kDa (Tables 1 and 2). Due to the higher DH, a 10 kDa MWCO membrane was utilized to optimize the fractionation process and the 10 kDa retentate of 10 % DH hydrolysate had ~50 % soluble material > 30 kDa (Table 2). Also, hydrolysis of heat-treated WPI to 10 % DH resulted in an increase in the percentage soluble material to 86 % (results not shown). The most noteworthy difference between the 10 % DH hydrolysates of the control and heat-treated WPI was the increase, by a factor of ~1.12, in soluble material < 1 kDa in the heat-treated 1 kDa permeate compared to the corresponding control permeate. Hydrolysis of the heat-treated WPI resulted in an increase in the percentage soluble material to ~57 % (results not shown). These results are in agreement with a previous study on the molecular mass distribution of aggregated dispersions which were hydrolysed using a similar laboratory scale process (O'Loughlin et al., 2013b).

3.2 ACE inhibitory activity of WPI, pilot-scale hydrolysates and associated membrane processed fractions

Samples (10 g L^{-1}) of control and heat-treated WPI, their respective 5 and 10 % DH hydrolysates and the associated membrane processed fractions were analysed for ACE inhibitory activity. The chromatographically determined changes in hippuric

acid formation (Fig. 2) in the presence of the 5 kDa retenate of the control 10 % DH hydrolysate are illustrated. Figure 2 also includes the inhibition profile for Captopril® (inset Fig. 2). Heat-treatment of WPI increased ACE inhibitory activity by, on average, a factor of 2.8 compared to the unheated control. The higher levels of hydrolysis (10 % DH) resulting in a greater increase in ACE inhibitory activity (~ 73 % at 10 g L^{-1}, Table 3) concurs with previous findings (Otte, Shalaby, Zakora, Pripp, & El-Shabrawy, 2007).

Higher IC_{50} values, and lower potency ACE inhibition was observed following concentration of higher M_w peptide material in the 30/10 kDa and 0.14 μm retentates compared to the un-fractionated whole hydrolysates. Correspondingly, the lowest IC_{50} value (0.23 ± 0.07 g protein L^{-1}) was observed for the 1 kDa permeate of heat-treated WPI hydrolysed to 10 % DH (Table 3). However, while generally the lower M_w fractions possessed the highest ACE inhibitory activity, the correlation between average M_w and inhibition activity was poor ($r = 0.524$, $P < 0.01$, results not shown). This was exemplified in the case of the 5 kDa retentate fractions from the control and heat-treated hydrolysates, which, while possessing similar M_w distributions (Table 1), presented significant ($P < 0.05$) differences in ACE inhibitory activity (Table 3). From Table 3, the 5 kDa retentate fraction from the hydrolysate of heat-treated WPI possessed an IC_{50} of 2.63 g protein L^{-1} – a reduction by a factor greater than 7 in the IC_{50} compared to respective fraction of the control hydrolysate (IC_{50} = 18.91 g protein L^{-1}). Also, this 5 kDa retentate fraction of the control 5 % DH hydrolysate represented the highest ACE IC_{50} (least potent) value observed from the tested spray-dried powders (Table 3). Mullally et al. (1997) demonstrated increased ACE inhibition in the 3 kDa permeates of WPC / β-lg tryptic hydrolysates compared to the whole un-fractionated hydrolysates (Mullally et al., 1997). However, from that study, the 1 kDa

permeates possessed lower ACE inhibitory activities than the 3 kDa permeates of the respective hydrolysates. The cumulative beneficial effect of both prior heat-treatment and higher DH is highlighted by the 10 % DH hydrolysate of the heat-treated WPI which had the highest ACE inhibition of the un-fractionated hydrolysates assayed (76.2 ± 1.2 %, Table 3).

3.3 Total iron and ferrous chelating capability of WPI, hydrolysates and fractions

Total iron content of the control WPI starting material (~ 228 μg L^{-1}) was in accordance with published values (Hunt & Meacham, 2001). Partitioning during hydrolysis treatments and fractionation resulted in values ranging from 38 ± 8 μg L^{-1} for the 1 kDa permeate (at 1 g L^{-1}) of the unheated control (10 % DH) to 419 ± 5 μg L^{-1} for the 30 kDa retentate of the unheated control (5 % DH) (results not shown).

The ferrous chelating capability, determined at 1 g L^{-1}, exhibited significant differences ($P < 0.05$) between the samples (Tables 3) with hydrolysis resulting in an increase in the chelating capability of WPI by a factor of ≥ 2. Generally, greater iron chelation (~ 55.7 %) was measured in the lower M_w fractionated samples. The ferrous chelating capability was higher for the 10 % DH hydrolysates compared to equivalent 5 % DH samples, irrespective of prior heat-treatment (Table 3). Interestingly, the whole hydrolysates of the heat-treated WPI samples had a lower percentage chelating ability (at 1 g L^{-1}) than equivalent unheated control hydrolysates at both 5 and 10 % DH. Overall, the highest percentage iron chelating ability (~ 55.7 %) was observed in the 1 kDa permeate of the 10 % DH heat-treated WPI (Table 3).

The concentration of sample required for the chelation of 50 % of ferrous iron under the assay conditions (CC_{50}) followed a similar trend as those seen in the % chelating ability. The lowest CC_{50} values were observed for the 1 kDa retentates and

permeates of both the control and heat-treated WPI hydrolysates (Table 3). Generally, lower CC_{50} values were seen with the 10 % DH samples and there appeared to be no discernable effect of prior heat-treatment of the substrate on iron chelation. While the 1 kDa permeates of the 5 % DH hydrolysates subjected to heat-treatment possessed lower iron CC_{50} values, in the 10 % DH samples the difference was not statistically significant ($P < 0.05$). Overall, the highest iron CC_{50} value was in the 30 kDa retentate of the unheated control 5 % DH hydrolysate (4.50 g L^{-1}), with the lowest value in the 1 kDa permeate of the heat-treated 10 % DH hydrolysate (0.83 g L^{-1}, Table 3).

There was a strong positive correlation ($r = 0.918$, $P < 0.01$) between the average M_w and the concentration needed to chelate 50 % of the iron (CC_{50}) in solution (Fig. 3) – average M_w being determined through integration of SEC size distribution data based on the area under the curve. For example, samples possessing an average M_w of ≤ 20 kDa possessed an equivalent CC_{50} value of ≤ 2.68 g L^{-1} while samples possessing an average M_w of < 1 kDa had CC_{50} values ≤ 0.86 g L^{-1}. Etcheverry et al. (2004) demonstrated higher iron chelating abilities with a 10 kDa permeate of whey compared to non-fractionated whey indicative of the presence of one or more low molecular mass Fe-binding components (Etcheverry, Miller, & Glahn, 2004). Another previous study demonstrated improved iron absorption in cellular models using hydrolysates of WPC with $M_w \leq 10$ kDa (Ou et al., 2010).

4. Conclusions

This pilot-scale study validates the findings of earlier laboratory experiments demonstrating altered kinetic, molecular and physico-chemical characteristics of hydrolysates obtained following pre-hydrolysis heat-treatments on WPI. Of particular significance was the enhancement of ACE inhibitory activity present in WPI

hydrolysates. This study demonstrated an increased ACE inhibitory activity as a direct result of prior heat-treatment of the WPI substrate and enrichment of ACE inhibitory activity in the lower M_w fractions. The ferrous chelating capability of the hydrolysate fractions was directly correlated with the average M_w of the peptides within the samples. There is a possibility that the bio-functionalities of these samples reflect the cumulative effect of more than one peptide in the fraction. Future work is required in the isolation and characterisation the active ingredients within these hydrolysates and fractions. Nevertheless, this work demonstrates the process scale up of thermally-denatured whey protein hydrolysis highlighting the concomitant consequences for downstream bio-functionality.

5. Appendices

5.1 Appendix A

The process flow diagram for the heat-treatment, enzymatic hydrolysis and subsequent membrane cascade fractionation of heat-treated (80 ºC for 10 min) WPI hydrolysed to 10 % DH is shown in Fig. A. 1.

5.2 Appendix B

Total solids and final volume determinations for all four processes are exhibited in Fig. B. 1.

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Figure 1. Hydrolysis profiles for whey protein isolate (WPI) unheated control and heat-treated (HT) dispersions (100 g L^{-1}) hydrolysed at pH 8 and 50 ºC to degree of hydrolysis (DH) values of 5 or 10 % with Corolase® PP obtained using the pH-stat methodology at pilot-scale (sample mean ± SD, $n = 2$).

Figure 2. Reversed-phase HPLC chromatogram showing the decrease in the formation of hippuric acid (from a single run quantified at 228nm) as a result of increased angiotensin-I-converting enzyme (ACE) inhibition for 5 kDa rentenate of the control 10 % DH hydrolysate. (Inset) ACE inhibition index determined with 6 – 24 nM of Captopril® where 15.4 nM Captopril® led to 50 % inhibition (IC_{50}) of ACE activity.

Figure 3. Correlation curve of the average molecular weight (M_w) of whey protein isolate, hydrolysates and fractions and their respective concentrations required to chelate 50 % of the ferrous iron from $FeCl_2$ (CC_{50}). Pearson's r value was determined to be 0.918 at $P < 0.01$.

Figure A. 1. Process flow diagram of heat-treated (80 ºC for 10 min) whey protein isolate (WPI) hydrolysed to a degree of hydrolysis (DH) of 10 % and subsequently micro- and ultra-filtered via membrane cascade fractionation. Where * = total solids, ** = solubility and *** = nominal molecular weight cut-off.

Figure B. 1. Total solids and final volume determinations for heat-treated (80 ºC for 10 min) and un-heated control whey protein isolate (WPI) hydrolysis processes where; A: control WPI 5 % degree of hydrolysis (DH), B: heat-treated WPI 5 % DH, C: control WPI 10 % DH and D: heat-treated WPI 10 % DH.

Figure 1.

△ = control 5 %DH, ◆ = HT 5 %DH, □ = control 10 %DH, ▲ = HT 10 %DH

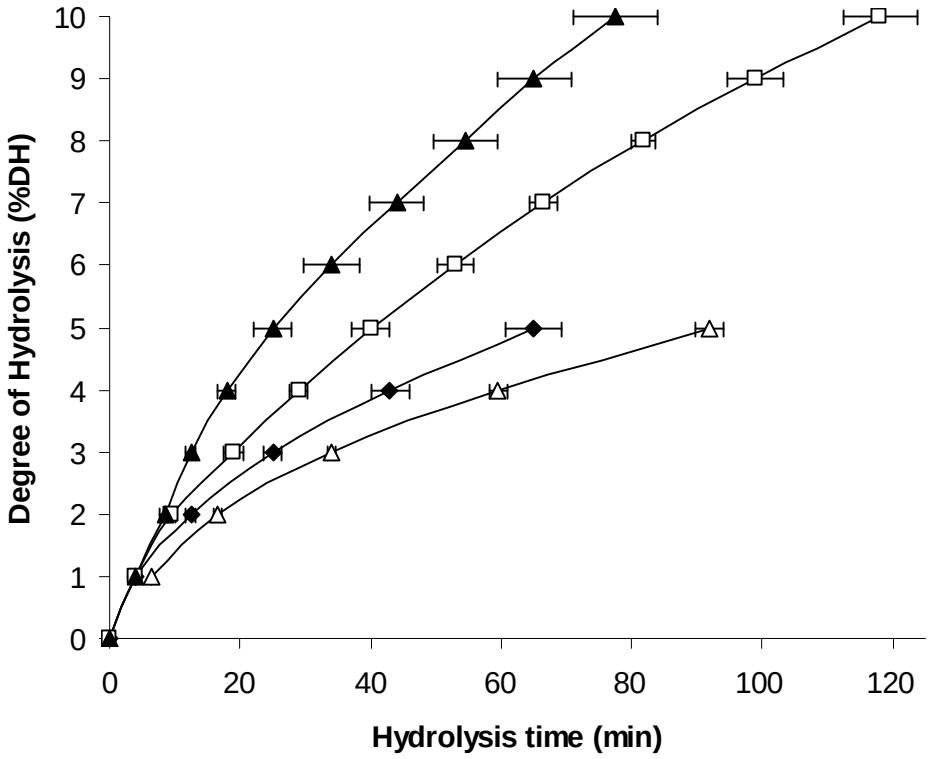


Figure 2.

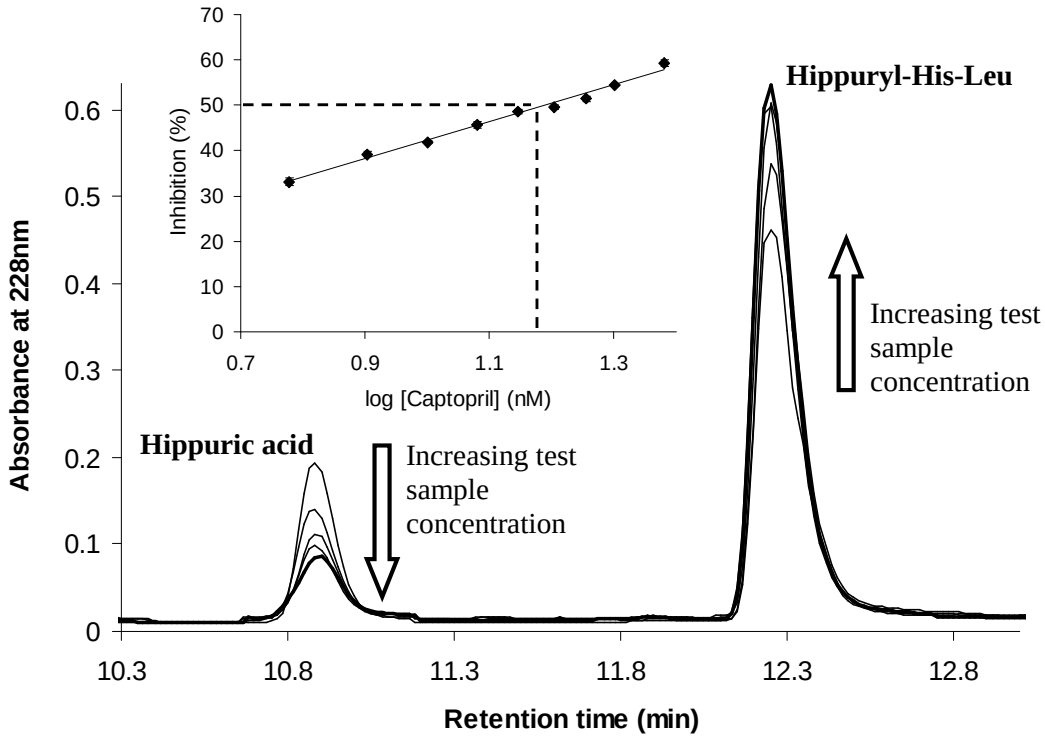

Absorbance at 228nm
Retention time (min)
Hippuric acid
Hippuryl-His-Leu
Increasing test sample concentration
Increasing test sample concentration
Inhibition (%)
log [Captopril] (nM)

Figure 3.

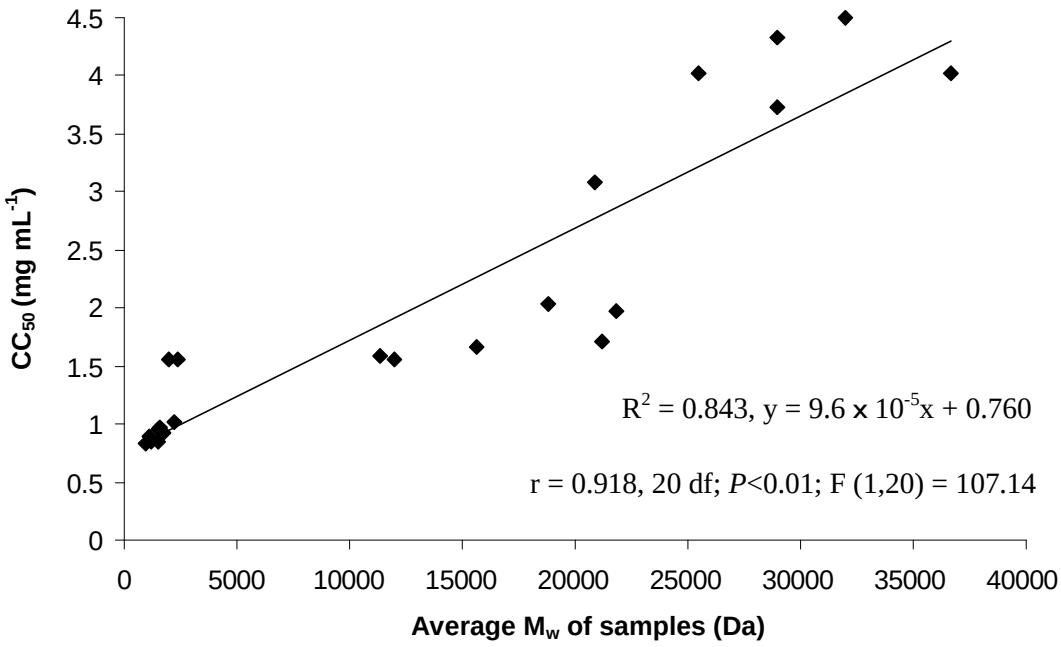

4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
5000
10000
15000
20000
25000
30000
35000
40000
CC50 (mg mL-1)
Average Mw of samples (Da)
R2 = 0.843, y = 9.6 x 10-5x + 0.760
r = 0.918, 20 df; P<0.01; F (1,20) = 107.14

Figure A. 1.

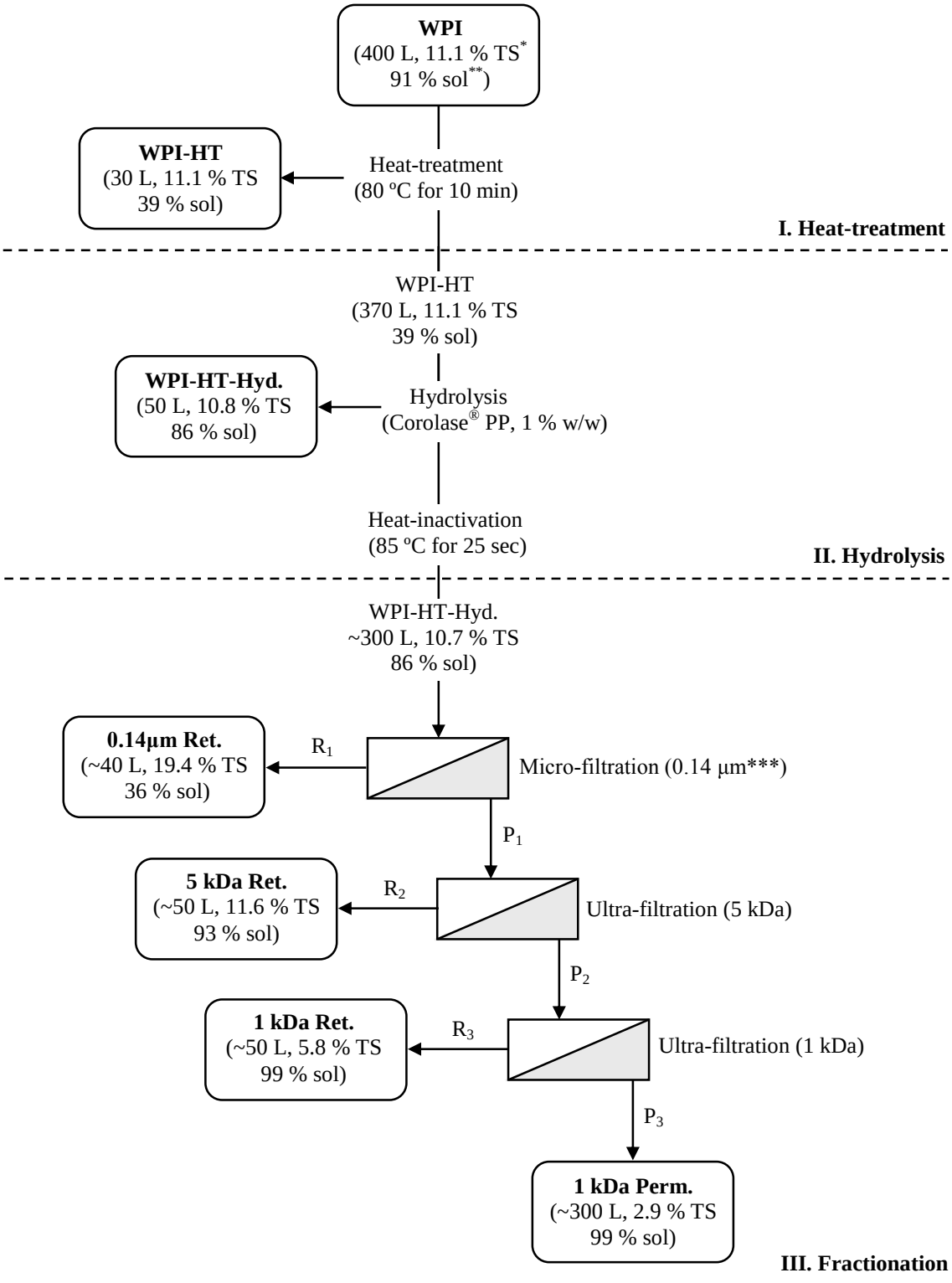
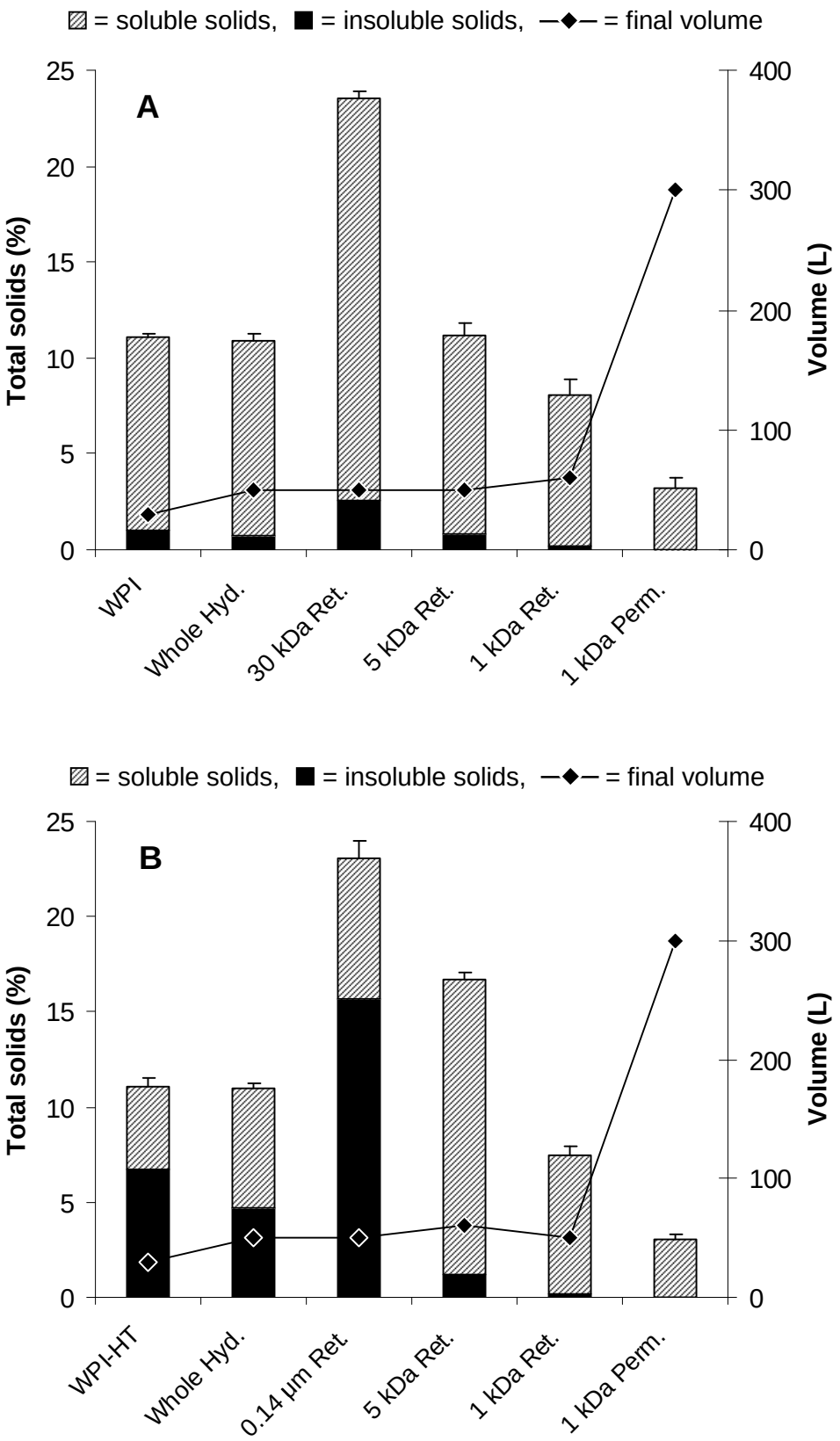
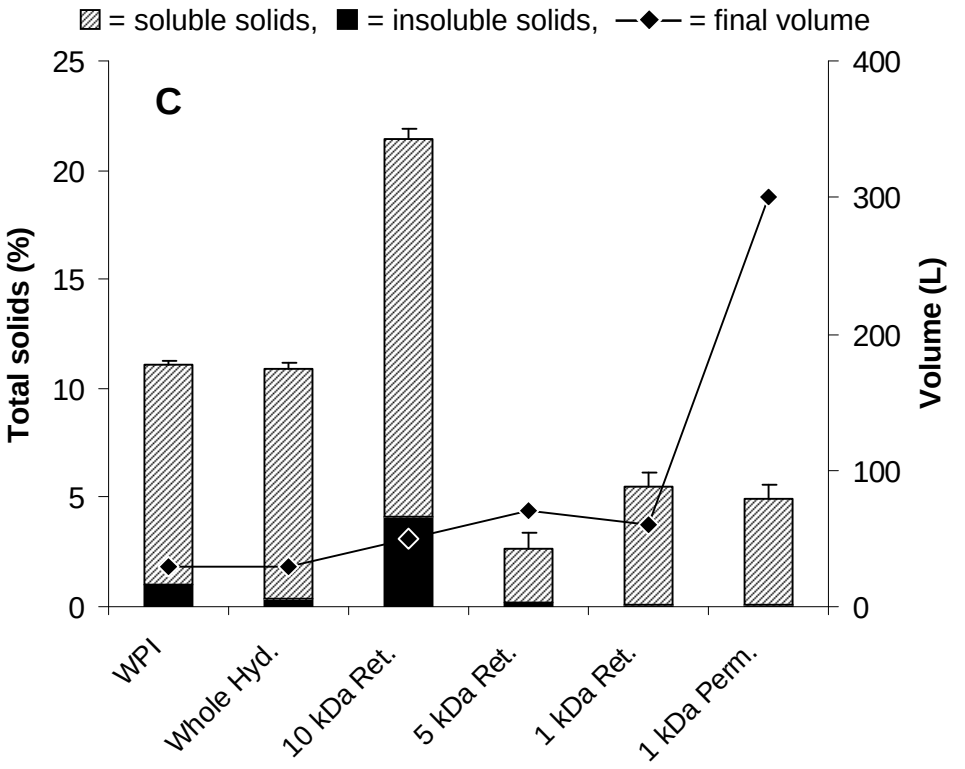
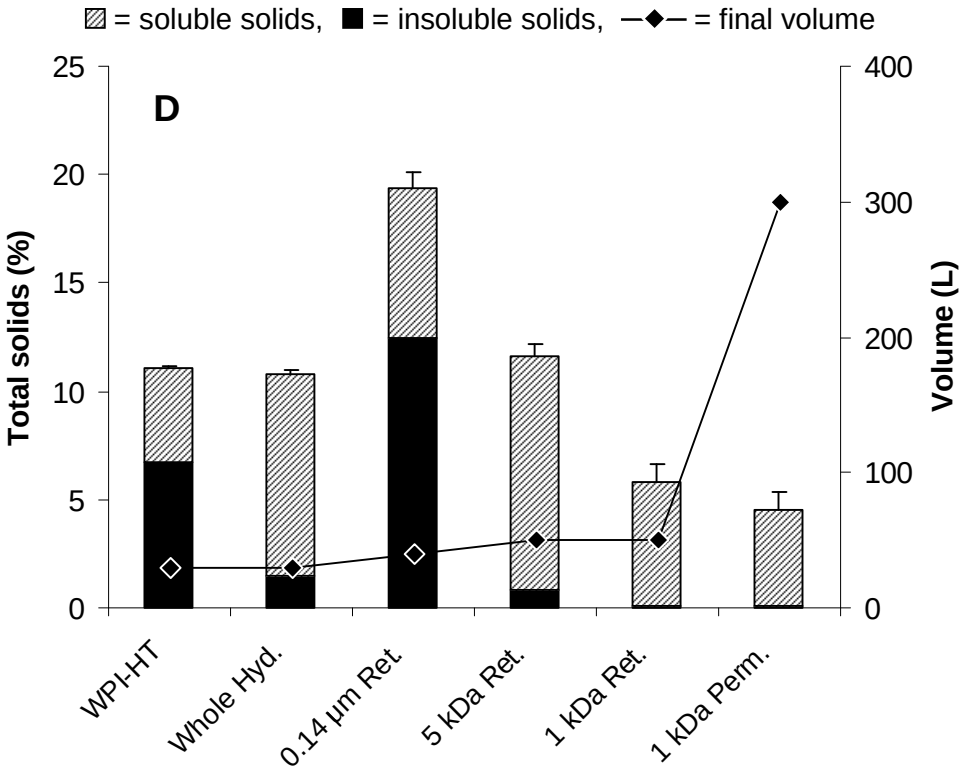

WPI
(400 L, 11.1 % TS*
91 % sol**)
WPI-HT
(30 L, 11.1 % TS
39 % sol)
Heat-treatment
(80 ºC for 10 min)
I. Heat-treatment
WPI-HT
(370 L, 11.1 % TS
39 % sol)
WPI-HT-Hyd.
(50 L, 10.8 % TS
86 % sol)
Hydrolysis
(Corolase® PP, 1 % w/w)
Heat-inactivation
(85 ºC for 25 sec)
II. Hydrolysis
WPI-HT-Hyd.
~300 L, 10.7 % TS
86 % sol)
0.14µm Ret.
(~40 L, 19.4 % TS
36 % sol)
R1
Micro-filtration (0.14 µm***)
P1
5 kDa Ret.
(~50 L, 11.6 % TS
93 % sol)
R2
Ultra-filtration (5 kDa)
P2
1 kDa Ret.
(~50 L, 5.8 % TS
99 % sol)
R3
Ultra-filtration (1 kDa)
P3
1 kDa Perm.
(~300 L, 2.9 % TS
99 % sol)
III. Fractionation

Figure B. 1.


▨ = soluble solids, ■ = insoluble solids, —◆— = final volume
A
Total solids (%)
Volume (L)
0
5
10
15
20
25
0
100
200
300
400
WPI
Whole Hyd.
30 kDa Ret.
5 kDa Ret.
1 kDa Ret.
1 kDa Perm.
▨ = soluble solids, ■ = insoluble solids, —◆— = final volume
B
Total solids (%)
Volume (L)
0
5
10
15
20
25
0
100
200
300
400
WPI-HT
Whole Hyd.
0.14 µm Ret.
5 kDa Ret.
1 kDa Ret.
1 kDa Perm.


▨ = soluble solids, ■ = insoluble solids, —◆— = final volume
C
Total solids (%)
Volume (L)
25
20
15
10
5
0
400
300
200
100
0
WPI
Whole Hyd.
10 kDa Ret.
5 kDa Ret.
1 kDa Ret.
1 kDa Perm.


▨ = soluble solids, ■ = insoluble solids, —◆— = final volume
D
Total solids (%)
Volume (L)
25
20
15
10
5
0
400
300
200
100
0
WPI-HT
Whole Hyd.
0.14 µm Ret.
5 kDa Ret.
1 kDa Ret.
1 kDa Perm.