Production of whey-derived DPP-IV inhibitory peptides using an enzymatic membrane reactor

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Abstract

Continuous processes for the production of peptides with specific bioactivity (PWSB) is an area of increased interest. In this study an enzymatic membrane reactor (EMR) was developed whereby whey protein isolate was used as a substrate to prepare DPP-IV inhibitory and radical scavenging peptides via enzymatic hydrolysis. Two separate enzymes were tested: Corolase 2TS and Protamex in conventional batch processes and the EMR. Neither enzyme was considered effective at producing peptides with radical scavenging activity when measured using a DPPH assay. However, both enzymes were capable of producing DPP-IV inhibitory peptides. Corolase and Protamex both produced similar DPP-IV inhibition levels upon completion of batch experiments. In the EMR process, permeate in the Protamex run showed 33.7% lower IC50 value compared to the continuous Corolase run. Protamex was a better enzyme at producing the DPP-IV inhibitory effect. The continuous (EMR) production method showed an increased productivity over batch for both enzymes.

Key Words: Enzymatic membrane reactor; DPP-IV inhibitory peptides; Peptides with specific bioactivity; Whey protein

1. Introduction

The importance of nutrition in the role of fetal and infant development, sport performance, healthy aging, apetite modulation and reduction of oxidative stress in the human body cannot be understated. Bovine milk can play a critical role in providing the nutrients and resources a body needs while under varying stresses and conditions. In addition to accounting for daily protein intake, proteins in milk have multiple functional roles and some have potential protective effects in health. Type 2 diabetes (T2D) is a growing problem. Control of blood glucose has been observed in human intervention studies with food proteins and food protein hydrolysates (Zhu, Li et al. 2010, Manders, Hansen et al. 2014, Méric, Lemieux et al. 2014). It is believed that dietary amino acids and short chain peptides may impact on T2D by inhibition of metabolic enzymes involved in the regulation of serum glucose (Nongonierma and FitzGerald 2016). Dipeptidyl peptidase IV (DPP-IV) is an ubiquitous enzyme which has been shown to cleave and inactivate GLP-1 and GIP in the postprandial phase, leading to a loss in their insulinotropic activity (Juillerat-Jeanneret 2013). Inhibition of DPP-IV can prolong the postprandial incretin effect, maintaining insulin secretion and helping reduce blood glucose. The most potent inhibitor of DPP-IV found to date is Diprotin A and is used as a benchmark for other DPP-IV inhibitors.

Whey proteins make up 20% of the protein in milk and whey is a coproduct of cheese manufacturing usually containing high amounts of lactose and salts, depending on the method of cheese production. The breakdown of whey protein varies but the generally accepted constituent components are beta-lactoglobulin (65%), alpha lactalbumin (25%), bovine serum albumin (BSA) (8%), and immunoglobulins (IGs) (1-2%) (Haug 2007). Due to their higher concentration, beta-lactoglobulin followed by alpha lactalbumin are of greatest interest when researching bovine milk proteins and peptides with specific bioactivities (PWSB). Whey protein has been studied for valorisation (Nath, Verasztó et al. 2016) and used in many instances to produce PWSBs and the list of bioactivities reported includes DPP-IV inhibition (Nongonierma and FitzGerald 2013, Nongonierma and FitzGerald 2013, Le Maux, Nongonierma et al. 2015, Le Maux, Nongonierma et al. 2015), antioxidant activity (Nongonierma and FitzGerald 2013, Le Maux, Nongonierma et al. 2016), and ACE-inhibition (Corrêa, Daroit et al. 2014, Lacroix, Meng et al. 2016).

PWSBs are produced as a result of proteolysis of a protein substrate by chemical or enzymatic means, or by bacterial fermentation. Within the native protein, bioativity is latentand becomes active upon release via hydrolytic cleavage. Chemical hydrolysis is the least favorable method due to restrictions of chemical use within the food industry as well as the use of environmentally unfriendly chemicals (Zambrowicz A. 2013). While bacterial

fermentation has been used successfully to produce PWSBs (Jemil, Jridi et al. 2014, Ha, Chang et al. 2015, Solieri, Rutella et al. 2015) some drawbacks remain including reduced specificity, longer production times and the production of undesired metabolites. Enzymatic production shows the best potential for up-scaling and commercialisation due to relatively fast processing times and mild processing conditions.

Enzyme immobilisation is a technique used to enable better control over the enzyme in hydrolysis processes and allowing it to be reused and/or recycled (Jakovetić, Luković et al. 2015, Nath, Verasztó et al. 2016, Erdős, Grachten et al. 2018). This can reduce cost while preventing further hydrolysis of PWSBs. Further hydrolysis of said peptides is undesirable as the bioactivity may be lost. Enzyme immobilisation techniques can also lead to elevated proteolytic activity over a longer period (Cabrera-Padilla, Pinto et al. 2009) by separating enzymes to prevent autolysis (Mateo, Palomo et al. 2007). However, strict immobilisation on a support matrix surface can be problematic, with difficulty attaching the enzyme to the support structure and reduced activity due to distortion of the enzyme because of interactions with the support (Rodrigues, Ortiz et al. 2013). Enzymatic membrane reactors (EMRs) are an emerging technology and can serve as a form of enzyme immobilisation and can involve binding of enzymes to larger support structures such as gel particles (Cabrera-Padilla, Pinto et al. 2009) or the use of free enzymes maintained upstream of the membrane (Cheison, Wang et al. 2006). EMRs work by retaining the enzyme and feed substrate on the retentate side while allowing the newly formed PWSBs to permeate through the membrane, generating a product stream with increaased bioactivity. In this study, the enzyme is introduced into the reaction vessel and retained upstream of the membrane on the retentate side of the membrane, while being free and not bound to the membrane. EMRs have been previously studied for the hydrolysis of various proteins with investigations of fouling, flux, enzyme kinetics, amino acid sequence of peptides generated and process modelling (Cheison, Wang et al. 2006, Cabrera-Padilla, Pinto et al. 2009, Eisele, Stressler et al. 2013). However there are limited studies that examine the production of specific bioactive peptides in an EMR while also monitoring bioactivity *in vitro*. An EMR-type setup has been used specifically to produce peptides with DPPH activity using egg white as a substrate (Jakovetić, Luković et al. 2015) However there have been no studies on whey-derived DPP-IV bioactive peptides produced continuously in an EMR at the time of writing. A study carried out (Cabrera-Padilla, Pinto et al. 2009) focused on generating ACE-inhibitory and antioxidant peptides while using casein as a substrate doing so in an EMR with some success. It was shown that the same substrate and enzyme, used under the same conditions, in a batch system led to feedback inhibition of the *Bacillus lentus* alkaline peptidase by its hydrolysed peptides, whereas using an EMR avoided this and increased productivity by 28%.

The continuous production of bioactive peptides holds significant advantages over batch production methods. Among the benefits are lower energy consumption due to reduced processing time, less unit operations, the potential for reduced enzyme usage leading to lower costs, and greater capacity per unit of floor space used in production facilities. However, there are some challenges that must be overcome or controlled to some extent. These include microbial proliferation, membrane fouling and reduced enzyme activity over time. It is also important to ensure that the process conditions are optimised for the enzyme being used, while remaining compatible with the membrane. If the optimal conditions for BP production vary from those for optimal fractionation, a trade-off will need to be made between the two in order to ensure the process remains efficient.

The aim of the present study was to establish an improved method for the production of whey-derived DPP-IV and antioxidant peptides by a direct comparison with a standard batch production method. An improved production method also allows for quick production of peptides which can then be tested for bioactivity using relevant assays. Using 2 commercially produced enzymes and 2 assays to check for DPPH and DPP-IV inhibition, a direct comparison between batch and continnuous methods is made.

2. Materials and Methods

2.1 Materials

Whey protein isolate (WPI) was supplied by Carbery Group, Cork. Corolase 2TS was donated by AB Enzymes GmbH, Germany. Protamex was purchased from Novozymes, Denmark. Polyethersulfone (PES) membrane sheets with a molecular weight cut off (MWCO) of 3kDa were supplied by Synder Filtration, USA in 1m^2 sheets and were stored at 4°C until required, and were then cut to size (0.0092m^2) using a stanley blade. DPP-IV enzyme was supplied by MerckMillipore, Ireland. NaOH, HCl, EtOH, MeOH, Tris, p-Nitroanalide, Gly-Pro-P-Analide, DPPH, Trolox, Selenium Kjeldahl digestion tablets, H_2SO_4, KOH, Boric Acid were all supplied by Sigma Aldrich, Ireland.

2.2 Choice of Processing Conditions

Choice of process conditions depended on the optimisation of both enzyme kinetics and membrane fractionation. A number of preliminary experiments were conducted to confirm the optimal enzymatic conditions laid out in the manufacturers guidelines. Firstly, the

enzyme manufacturers recommendations were considered for temperature, pH and dosing. Membrane suppliers guidelines were also considered, in the case of processing temperature.

Temperature was chosen (55ºC) to maximise flux while remaining within recommended processing temperatures by both enzyme manufacturers. pH was chosen (7.5) based on flux experiments and enzyme manufacturers recommendations. Enzyme dosing was based on upper limit recommendations by enzyme manufacturers to avoid enzyme dosing being a limiting factor in hydrolytic activity. They were 25mg Protamex/kg of substrate and 50ml Corolase/kg substrate.The same processing conditions were used for all runs.

Manufacturers guidelines for optimum conditions for Protamex were: Temp=35ºC-60ºC, pH=5.5-7.5, dosing=substrate dependant. For Corolase: Temp=45ºC-75ºC, pH=6.0-9.0, dosing=substrate dependant. Batch hydrolysis experiments were carried out to confirm that rate of hydrolysis were maximum at the following conditions for Protamex: Temp=40ºC, pH=6.0. For Corolase: Temp=65ºC, pH=7.5. The rate of hydrolysis slowed when moving away from these optima. Runs were carried out to compare kinetics at operating conditions and manufacturers optima (data not shown). Kinetics were determined by graphing %DH versus time in all cases. %DH was calculated using pH-stat method in all cases.

2.3 Cross-Flow UF System and Procedure

The cross-flow UF system and associated instrumentation was built in-house. The system was equipped a high pressure, metering pump (model P200, Hydra-Cell, UK). The experimental set-up can is shown in Figure 1.

A moderate cross-flow velocity was chosen (~0.5m/s) to minimise foaming and membrane fouling. A high trans-membrane pressure of 6 bar was chosen to ensure a relatively high flux,while a 3kDa MWCO membrane was chosen to fractionate smaller peptides, while not reducing the flux to unworkable rates. The enzymes were carefully introduced into the reaction vessel at the beginning of their respective incubation periods.The degree of hydrolysis (DH) was monitored during the incubation period as described elsewhere in this section. Upon completion of a 40 minute incubation period, the three pumps were started, initiating the membrane separation process and fresh feed in-flow. This incubation period ensured a %DH of between 6 and 9%. Temperature, pressure, retentate flow rate, cross flow velocity and flux were all monitored throughout the process. Two 50ml samples were taken every hour to measure protein content, check for bioactivity (DPP-IV and DPPH). Eight hours after separation commenced, the pumps were stopped and pure water fluxes (PWFs) were checked after rinsing and then again after a full cleaning regime, as described elsewhere in

this section. Enzyme leakage was checked by monitoring pH in permeate samples over a 4 hour period while samples were maintained at 25°C. If enzyme activity was still present in samples via leakage through the membrane, a reduction in pH would be measurable due to further hydrolysis. This is the basis of the pH-stat method for measuring the degree of hydrolysis.

2.4 Batch Runs

For the batch experiments, 5L vessels were used with temperature control at 55°C. The vessels were stirred at 300rpm with an overhead paddle stirring system. There was a limited amount of foaming observed. 3.5L of WPI solution at a concentration of 5% w/w (protein basis) was made up. Once the water bath and WPI solution were at a steady temperature of 55°C, the pH was adjusted to 7.5 using 2M NaOH and the enzyme was added at the appropriate enzyme to substrate ratio (1:100 v:w for Corolase and 1:100v/v Protamex, protein basis). The timer was started as soon as the enzyme was added and the pH was maintained at 7.5 by manually adding 2M NaOH. The mass of NaOH added was recorded at 2 minute intervals and samples of the hydrolysate were taken every six minutes for the first 30 minutes, every 10 minutes for the following 30 minutes and every half hour for the next three hours, with a total experimental time of four hours. The enzyme was deactivated by heating the samples to 80°C for five minutes in a water bath. The samples were stored at -20°C and utilised within 72 hours for all necessary testing. Permeate samples were also tested for enzyme leakage.

2.5 EMR Runs

The cross-flow system, pump and piping was cleaned with NaOH followed by nitric acid and finally rinsed with 70% MeOH prior to intalling the membrane. Membrane sheets were cleaned with MilliQ water prior to fitiing in the cross-flow cell. Pure water flux (PWF) measurments were undertaken to ensure the variation in pore size and pore density was not greater than 20%. All PWF runs were carried out at 25°C to ensure consistent viscosity, while the transmembrane pressures were also kept constant at 7bar. A cross-flow velocity of 0.5m/s was controlled via pump speed settings. PWFs were measured over two minute intervals by collecting and weighing the permeate. This was done until the variation between readings was less than 5%. The system was then allowed to drain and the main experiment commenced by adding in WPI substrate solution followed by the enzyme, to the

reaction vessel. After the 40 minute incubation period, the permeate flux was measured every 60 minutes until the end of the experiment. Since a relatively low NMWCO was chosen, 3kDa membrane, it was important to ensure that the DH of the whey substrate was high enough to allow a high mass flux of peptides. The level of bioactivity at that time was also considered and was deemed to be approximately 90% of maximum bioactivity achieved in the DPP-IV batch bioassay. For these reasons an initial incubation period of 40 minutes was chosen for both enzymes in the EMR runs.

After the experimental runs, the system and membrane was rinsed with DI water and then rechecked for PWF. The system and membrane was then subject to a full cleaning regime, first using a mixture of hydrochloric and nitric acid at pH 2.8 and 50°C for 30 minutes followed by rinsing with MilliQ. Subsequently a caustic wash was performed using NaOH at pH 10.8 and 50°C for 30 minutes followed by rinsing with MilliQ. The PWF was then rechecked.

2.6 Mass Balance, Mass Flux and Protein Determination

Protein concentration was determined in all feedstock, permeate and retentate samples using the Kjeldahl method (ISO 2009). The protein content of both enzymes was also analysed. For the mass balance, the starting mass of protein and fresh feed added were measured directly. Retentate and permeate were measured for volume, mass and protein content using Kjeldahl method, while starting solution and feed were measured for protein using Kjeldahl also. Results of mass balance were verified by measurements (partial data shown). Mass flux of Nitrogen was determined by multiplying measured fluxes by Nitrogen percentage (measured by Kjeldahl). Protein/peptide flux was determined from this data using Kjeldahl conversion factor.

2.7 DPPH Assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) was dissolved in HPLC grade ethanol / MilliQ H_2O (70:30) and diluted to a concentration of 120µmol. Care was taken to ensure the DPPH solution was not exposed to light. Samples were prepared at a final concentration of 1mg/ml (protein basis). and were centrifuged at 5000g for 10 minutes prior to assay. Samples (0.5mL) were added to 1mL aliquots of the 120µmolDPPH solution,incubated in the dark at room temperature for 45 minutes and their absorbance at 517nm measured. All samples were analysed in triplicate and all absorbance readings were performed in triplicate

automatically for each sample also by the spectrophotometer,. The Radical scavenging Activity (RSA)% was calculated using Equation 1 below.

$$RSA\% = [(TB - TS)/TB]\, X\, 100 \qquad \textit{Eqtn. 1}$$

Where TB = test blank, TS = test sample

2.8 DPP-IV Inhibition Assay

The DPP-IV assay was carried out in triplicate as per Van Amerongen et al, (Aart, Catharina et al. 2009) with some variations. Samples were diluted to final concentrations of 0.5, 1, 1.5 and 2mg/ml using 100mM Tris-HCl buffer, pH 8. Aliquots (25µl) of sample were pipetted into a 96 well-plate and pre-heated to 37°C along with 25µl of Gly-Pro-P-nitroanilide substrate. Once pre-heated, 50µl of the DPP-IV enzyme, pre-diluted to the appropriate concentration based on its activity, was added to the well plate and the release of pNA determined in a Thermo Scientific Varioskan LUX Multimode Microplate Reader at 405nm . The activity of the DPP-IV enzyme preparation was confirmed prior to using it in the bioassay by assessing the amount of P-Nitroanilide released using Gly-Pro_P-Nitroanilide as a substrate. The enzyme activity deviated by less than 5% from the activity specified by the supplier. Absorbance readings were taken after two hours of incubation. Test blanks, positive control and negative controls were also analysed. The % inhibition was calculated as per Equation 2.

$$(1 - [(TS - TB)/(PC - NC)])\, X\, 100 \qquad \textit{Eqtn. 2}$$

Where TS = test sample, TB = test blank (no enzyme, PC = positive control and NC = negative control.

IC_{50} values were defined as the concentration of any product stream at which an inhibition of DPP-IV enzyme is observed during the DPP-IV inhibition assay and is grams of protein per ml of solvent (water). IC_{50} values not measured directly. Further details below in section 2.10.

2.9 Degree of Hydrolysis

Degree of hydrolysis was measured using the pH-stat methods according to (Adler-Nissen 1986) with some modifications. Briefly, 2M NaOH was used to maintain the protein solution and enzyme mix at pH 7.5 and the mass of NaOH solution added was recorded. Equation 3 below was used in the calculation.

$$DH = B\ X\ NB\ X\ 1/\alpha\ X\ 1/Mp\ X\ 1/Htot\ X\ 100 \qquad \textit{Eqtn. 3}$$

Where B = mass of base added, N_B = normality of base, a = average degree of dissociation, M_p = mass of protein, H_{tot} = total number of peptide bonds in protein (meqv/g).

The average degree of dissociation was based on (Adler-Nissen 1986). It has been noted that this value can change if the enzyme used is an exopeptidase, which has a propensity to cleave the peptide bonds at the ends of proteins and peptides, producing a greater amount of free amino acids, di- and tri-peptides (Spellman, McEvoy et al. 2003).

2.10 Comparison of Runs

An overall comparison of runs was carried out. The total mass of DPP-IV enzyme inhibited (calculated through use of the assay) by peptides generated was calculated per gram of feedstock used. Because there was no direct measurement of the concentration of PWSBs produced, this way was chosen to express a 'purity equivalent'. To make a fair comparison between the 2 production techniques, both the permeate and retentate from the EMR runs were used in this calculation, as there was still significant bioactivity in the retentate.

2.11 Statistics and Replication

Each of the 4 main runs were carried out in duplicate and results expressed as an average. Each sample measured by all techniques was made into triplicates and averaged unless otherwise stated. All spectrophotometer readings were carried out in triplicate. IC50 values for DPP-IV assay were carried out by 2 point linear interpolation using aggragates from triplicate analysis on Excel. Enzyme deactivition was measured by checking pH reduction 3 times over a 2 hour period in triplicate. DPPH assay results analysed using single factor ANOVA anlysis on Excel. Standard deviations were calculated for DPP-IV assay and Kjeldahl results on Excel.

3. Results and Discussion

3.1 Batch Experiments

The changes in DPPH quenching activity during batch hydrolysis for both enzymes are shown in Figure 2.

The enzyme reaction begins quickly with a steep increase in DH within the first 6 minutes and then slowing down somewhat while maintaining a steady rate of hydrolysis for the remainder of both reactions. Corolase had the faster enzyme kinetics of tge two as is evident from the higher DH, notable from minute 3. The general trends in RSA% results are similar for both Protamex and Corolase, showing a significant decrease (P-value = 0.012 and 0.026, respectively) in radical scavenging activity (RSA%) almost immediately upon commencement of hydrolysis (compared with non enzyme-treated substrate) with no significant further change therafter (σ = 1.47 and 1.77 respectively). There was also no significant difference between the two enzymes with respect to DPPH activity (P-value = 0.58). This reduction of RSA% is somewhat unexpected as whey has been used as a feedstock for production of antioxidative bioactive peptides in the past (Hernández-Ledesma, Recio et al. 2008, Peng, Xiong et al. 2009, Nongonierma and FitzGerald 2013, Brandelli, Daroit et al. 2015). A reduction in the activity upon hydrolysis suggests that there is no benefit to hydrolysis and that, if anything, it is detrimental to the antioxidant potential of WPI. The non-hydrolysed protein shows substantial RSA%, which has also been reported previously (Tong, Sasaki et al. 2000, Peng, Xiong et al. 2009). Although Protamex and Corolase 2TS show a reduction in RSA% upon hydrolysis, this does not necessarily mean that hydrolysis of whey protein will always lead to the same reduction and it has been shown elsewhere that RSA% increases with DH (Peng, Xiong et al. 2009, Peng, Kong et al. 2010, Kamau and Lu 2011). Despite no change in bioactivity with increasing DH, samples generated in the EMR were still analysed for RSA% for comparison to the batch hydrolysate. Again similar results were found in the continuous process using the EMR. Therefore the IC_{50} values with respect to DPPH were not determined.

Figure 3 shows the results of the DPP-IV bioassay for both enzymes used. The graph shows a positive correlation between the DH and bioactivity up until 120 minutes at which time the bioactivity appears to level off. At this point, bioactivity remains at ~37% for both batch runs.

The evolution of the bioactivity differs between the two enzymes with a higher level of bioactivity during the earlier stages (275% higher at 6 minutes and 35% at 12 minutes) of hydrolysis for Protamex, despite slower enzyme kinetics and lower DH. This emphasises the

point that DH is not necessarily the main driver or predictor of bioactivity and that enzyme type and specificity can have a greater impact on hydrolysate potency.

3.2 EMR Experiments

Figure 4 shows the performance of both EMR runs by plotting the mass flux of nitrogenous material over the bioactivity of the permeate streams during the course of the experiment.. Figure 4 shows that both the N mass flux and permeate bioactivity of declines over the run time in the case of hydrolysis by Protamex. This lower mass flux during Protamex runs can be explained by slower enzyme kinetics for the Protamex versus Corolase (figs 2 & 3), leading to a build-up of protein on the retentate side and fouling of the membrane. The flux shows a slow decrease over the first 4 hours (10%), followed by an accelerated decrease over the final 4 hours (33%). The final 4 hours also shows a resurgence in DPP-IV inhibition (potency) of the permeate product (10%).

With respect to Corolase 2TS, there is a relatively steady flux up until 6 hours. The protein content in the permeate stream reduces significantly after 6 hours (26.5% reduction in mass flux from 6 hours to 8 hours). Reduced enzyme kinetics after 6 hours, again leading to a build up of protein upstream of the membrane and subsequent fouling is the likely explanation for the reduction in overall and mass flux (Noble and Stern 1995, Van Reis and Zydney 2001, Seader and Henley 2011, Janson 2012).

In all EMR runs it was concluded that there was no enzyme leakage after random permeate samples were monitored for pH change as an index of enzyme activity as described in the materials and methods section.

3.3 Production Mass, IC_{50} and Cumulative DPP-IV Enzyme Inhibition

Table 1, shows a comparison between both continuous runs which was made by measuring IC_{50} values and the total mass of peptide produced. In the case of the continuous process using Corolase 2TS, the weighted average IC_{50} value including retentate was 2.601mg/ml whereas the value was 2.333mg/ml for EMR-Pro. When permeate alone is considered, the IC_{50} value for EMR-Pro was 33.7% lower than that of EMR-Cor. Thus the protamex run produced a more potent product. Consequently 11.5% more of the Corolase-generated product would need to be administered to achieve the same degree of DPP-IV inhibition. In terms of production, the mass of peptide produced was approximately 10.7% more using Corolase over Protamex. This was mainly due to better permeation during the Corolase run.

For a comparison between batch Vs. continuous production, while calculating productivity of the batch runs, only one IC_{50} value was considered and that is the IC_{50} of the sample taken

at 240 minutes while using Corolase 2TS and that taken at 180 minutes while using Protamex. These represent the reaction times at which the IC_{50} values were maximised for the respective runs. Protamex produced better DPP-IV activity throughout both the batch and continuous production compared to Corolase. However the higher flux rates during the Coroalse run gave a greater production of peptide in the permeate streams, on a mass basis. Given that the IC_{50} values are higher in the Corolase permeate streams, it is clear that less bioactive peptides were produced in this run, despite more peptides being produced.

Figure 5 shows the total DPP-IV enzyme inhibition per gram of substrate used (mg/g protein) as described in the methods and materials section. Although Corolase showed more production of permeate due to higher flux values, there was also more substrate used during that run for the same reason, as fresh feed replaced the permeate. Peptides for DPP-IV inhibition were produced in greatest quantity (when mass and IC_{50} were combined) during the continuous (EMR) Protamex run, followed by batch production using Protamex. For both enzymes, the EMR run out-performed the batch runs, showing the continuous method of production to be a more effective production method when maximising product mass output and producing a more potent product (lower IC_{50}). Protamex showed a 28.3% better overall PWSB production in batch runs using the metrics in table 1. The same metrics showed Protamex to be 26.5% better than Corolase on average during the continuous runs.

4 Conclusions

Corolase 2TS and Protamex were both shown to be effective enzymes in producing peptides with DPP-IV inhibitory affect using WPI as a substrate. Furthermore, a continuous method of production using an EMR proved to be a more efficient way of producing DPP-IV inhibitory peptides compared to standard batch processing methods. An increased production output of 7.2% and 8.7% was achieved in continuous processing versus batch for Protamex and Corolase respectively. Fluxes, specifically mass flux of protein fluctuated slightly for all continuous runs, which can be overcome by engineering a more accurate feed dosing system to ensure the total solids fed in (feed) versus out (permeate) are well balanced. Despite some small fluctuations, generally flux trends remained stable and relatively high, with the Protamex runs showing a slight but persistent reduction in flux over the entirety of the runs. When comparing average fluxes to PWFs, fluxes averaged between 25% - 45% of the PWFs aided in part by a cleaning effect on the membrane by the enzyme during processing.

391 Cleaning regimes proved to be very effective indicating that there was no permanent
392 membrane fouling as evidenced by restoration of PWF following cleaning.

393 The benefits of the EMR process include increased production of DPP-IV inhibitory peptides
394 per kg of feed and reduced capital expenditure upon scale-up compared to batch. It is likely
395 that enzyme usage would be reduced with the EMR process though this has not been
396 evaluated here. This processing method has applications in peptide production that utilises
397 enzymatic hydrolysis to produce an end product. Furthermore this process can be used in
398 other nutraceutical applications that also involve enzymatic hydrolysis of a substrate to
399 produce a desired product, such as prebiotic oligosaccharides.

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Figures

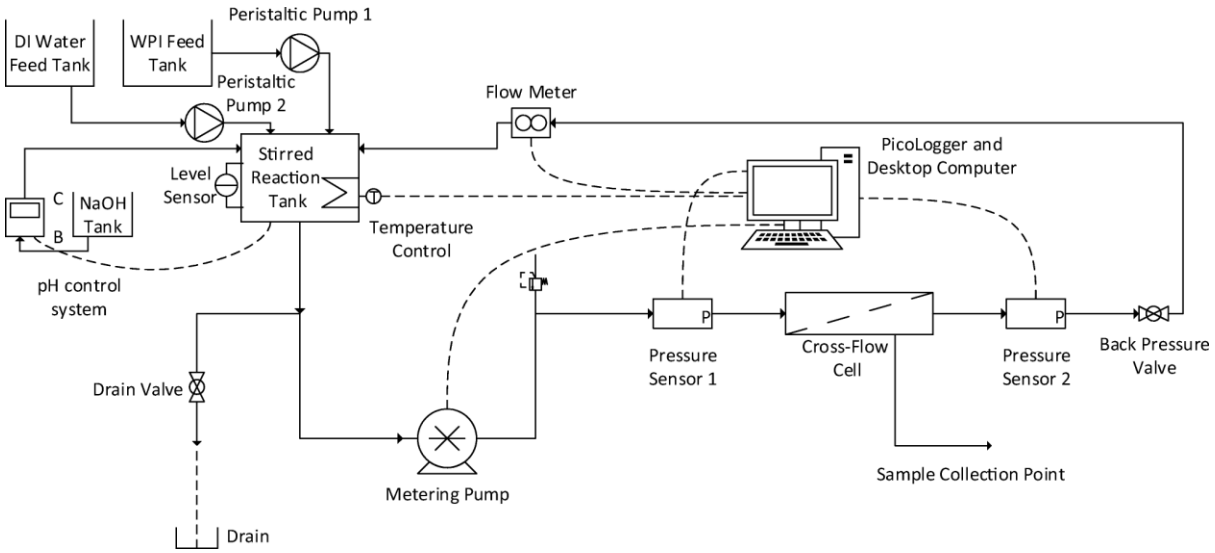


Figure 1. Experimental setup for the enzymatic membrane reactor (EMR) experiments. Feed Tank 1 contained deionised water and Feed Tank 2 contained 5% WPI solution. Both peristaltic pumps were controlled to adjust the concentration of WPI in the fresh feedstock entering the reaction vessel. After an incubation period, all three pumps were started along with flux, pH, temperature and pressure monitoring, and samples were taken at regular intervals.

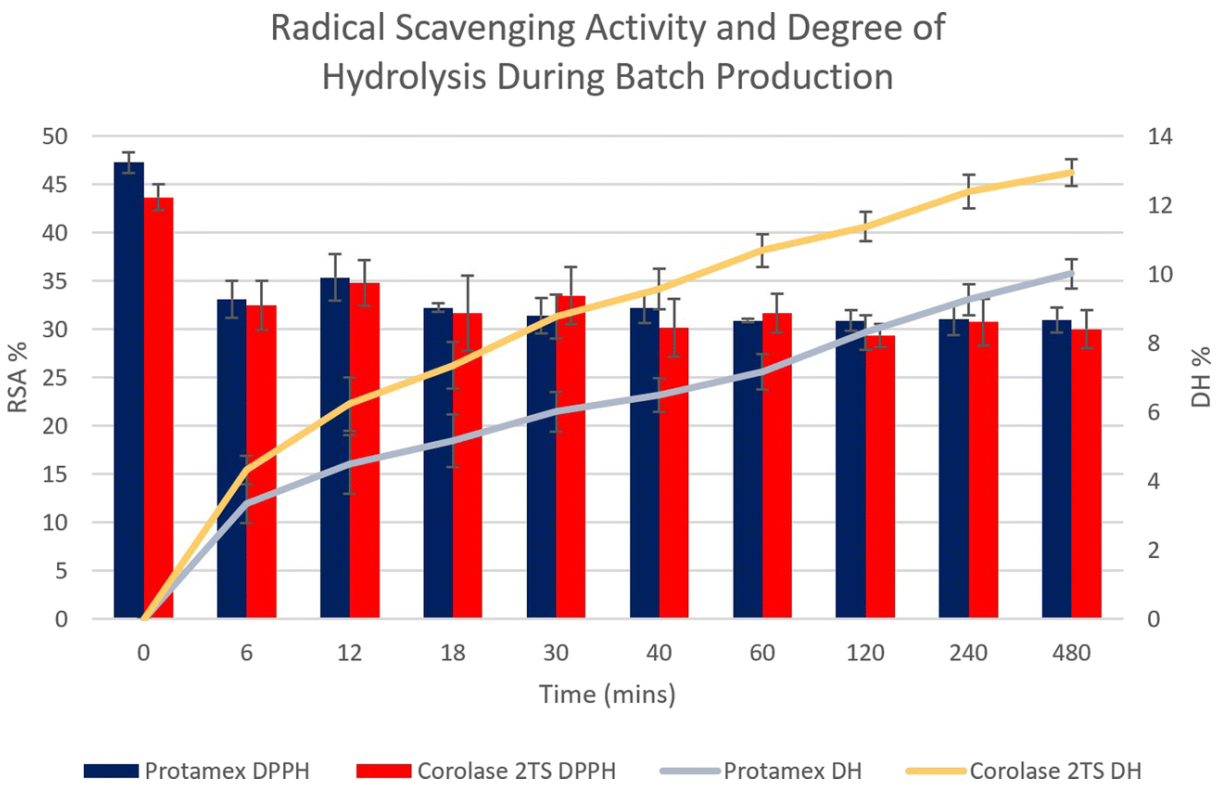


Figure 2: Evolution of Radical scavenging activity (RSA,%-bars) and degree of hydrolysis (DH,%-lines) during batch hydrolysis of WPI (5% protein basis) using Corolase 2TS, E:S = 1:100 (v/w protein basis) in red and Protamex, E:S 1:100 (w/w protein basis) in blue. Both batch experiments were carried out at 55°C while maintaining pH at 7.5 using 2M NaOH. Sample concentration in DPPH assay 1mg/ml.

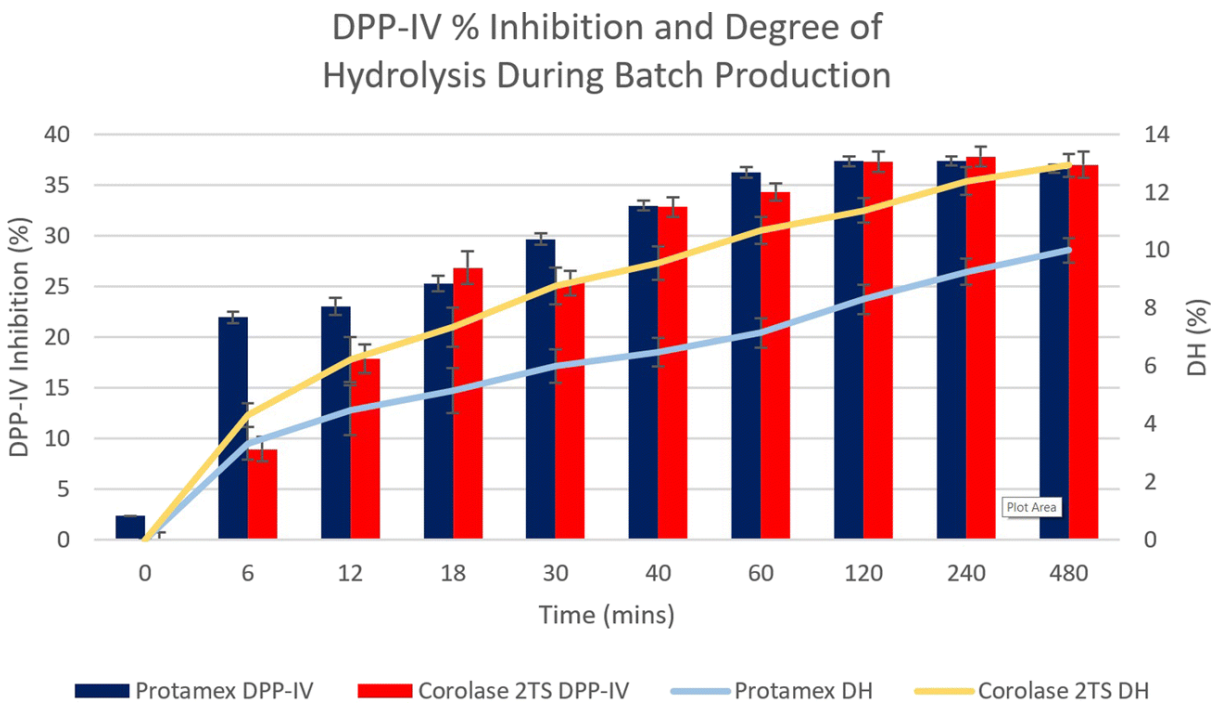


Figure 3: Evolution of DPP-IV inhibitory activity and DH during batch hydrolysis of 5% WPI (protein basis) using Corolase 2TS, E:S = 1:100 (v/w protein basis) in red and Protamex, E:S 1:100 (w/w protein basis) in blue. Both batch experiments were carried out at 55°C while maintaining pH-stat at 7.5 using 2M NaOH. All samples were measured at 1mg/ml final concentration (protein basis).

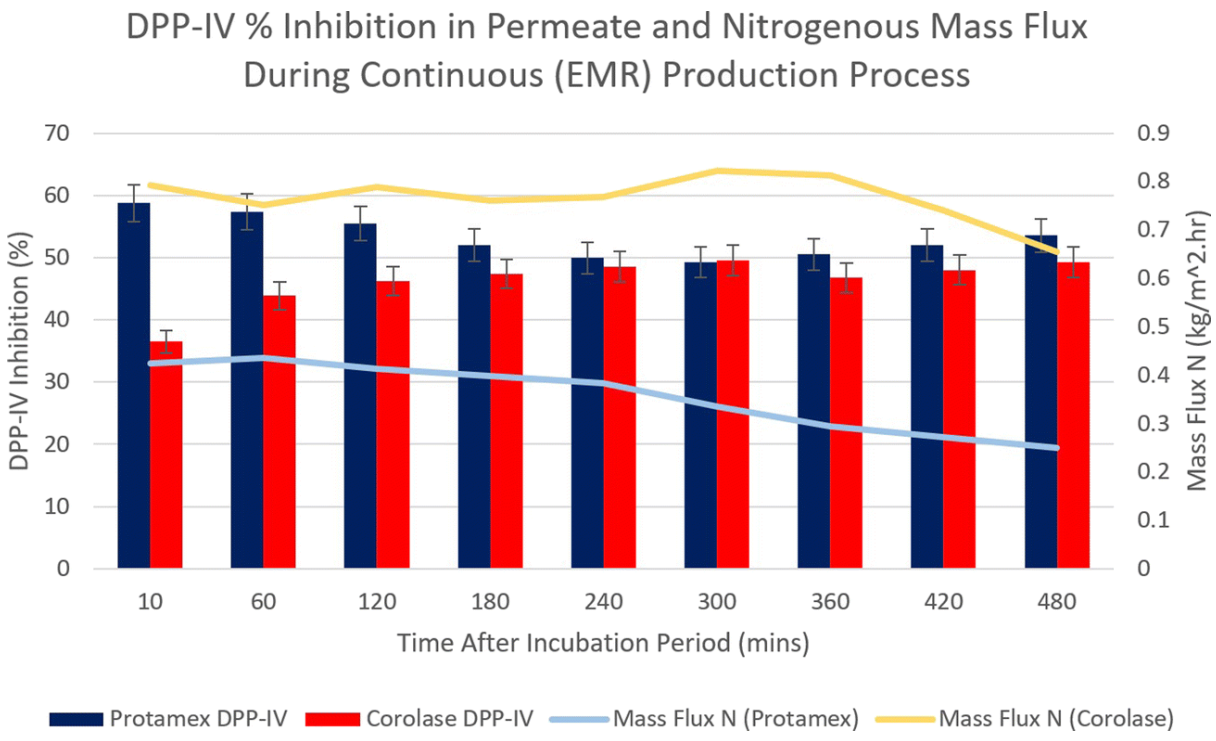
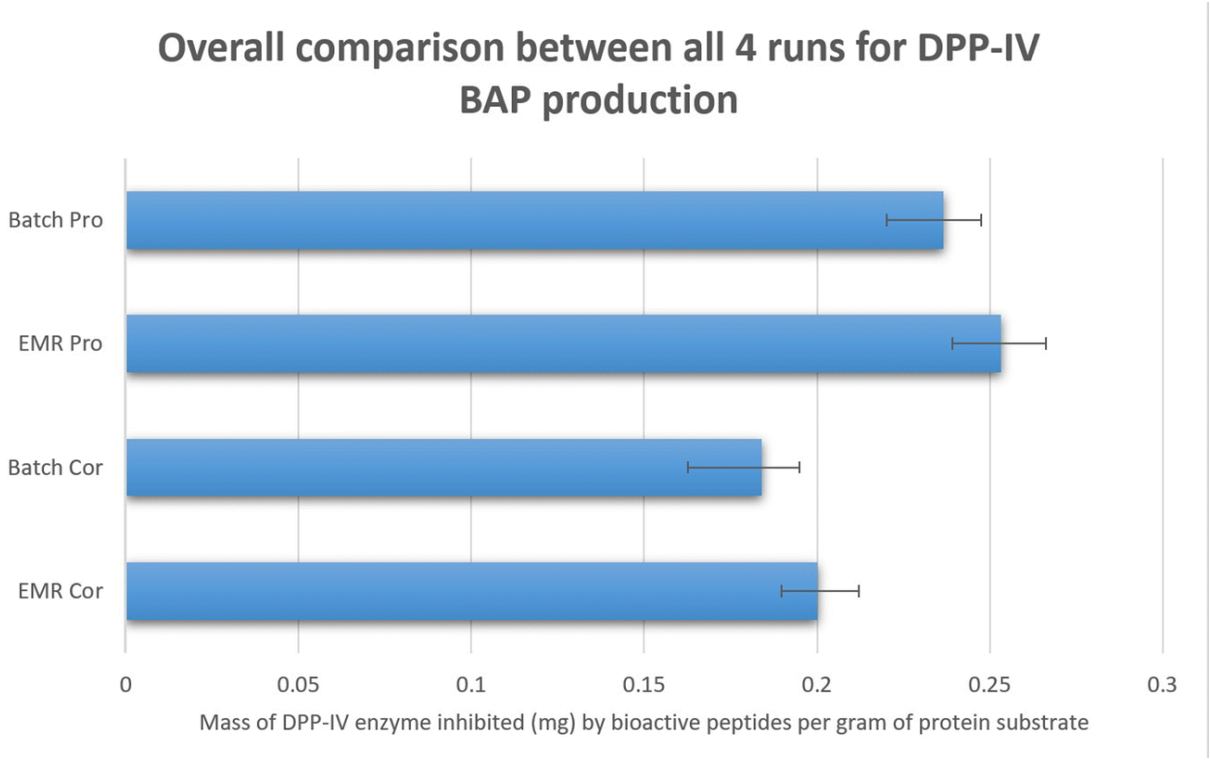


Figure 4: Changes in DPP-IV inhibitory activity of permeate and mass flux during EMR Runs 1 and 2 using Corolase 2TS (blue) and Protamex (red) respectively. Conditions were the same as the previous batch experiments. Hydrolysis was allowed to proceed for 40 minutes (incubation period) before the pump was switched on and CFUF separation began using a 3kDa PES membrane. All data generated after incubation period.



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546 **Figure 5**. A comparison is made between runs by taking into account IC_{50} values and mass produced.
547 By calculating the mass of DPP-IV enzyme inhibited by 50% in each assay, a total theoretical mass of
548 DPP-IV enzyme inhibited by 50% is calculated, enabling a direct comparison between the 4 runs.

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