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

3

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14

15 **Abstract**

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

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

30 1. Introduction

31 The importance of nutrition in the role of fetal and infant development, sport performance,
32 healthy aging, appetite modulation and reduction of oxidative stress in the human body
33 cannot be understated. Bovine milk can play a critical role in providing the nutrients and
34 resources a body needs while under varying stresses and conditions. In addition to
35 accounting for daily protein intake, proteins in milk have multiple functional roles and some
36 have potential protective effects in health. Type 2 diabetes (T2D) is a growing problem.
37 Control of blood glucose has been observed in human intervention studies with food proteins
38 and food protein hydrolysates (Zhu, Li et al. 2010, Manders, Hansen et al. 2014, Méric,
39 Lemieux et al. 2014). It is believed that dietary amino acids and short chain peptides may
40 impact on T2D by inhibition of metabolic enzymes involved in the regulation of serum
41 glucose (Nongonierma and FitzGerald 2016). Dipeptidyl peptidase IV (DPP-IV) is an
42 ubiquitous enzyme which has been shown to cleave and inactivate GLP-1 and GIP in the
43 postprandial phase, leading to a loss in their insulinotropic activity (Juillerat-Jeanneret
44 2013). Inhibition of DPP-IV can prolong the postprandial incretin effect, maintaining insulin
45 secretion and helping reduce blood glucose. The most potent inhibitor of DPP-IV found to
46 date is Diprotin A and is used as a benchmark for other DPP-IV inhibitors.

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

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

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

70 Enzyme immobilisation is a technique used to enable better control over the enzyme in
71 hydrolysis processes and allowing it to be reused and/or recycled (Jakovetić, Luković et al.
72 2015, Nath, Verasztó et al. 2016, Erdős, Grachten et al. 2018). This can reduce cost while
73 preventing further hydrolysis of PWSBs. Further hydrolysis of said peptides is undesirable as
74 the bioactivity may be lost. Enzyme immobilisation techniques can also lead to elevated
75 proteolytic activity over a longer period (Cabrera-Padilla, Pinto et al. 2009) by separating
76 enzymes to prevent autolysis (Mateo, Palomo et al. 2007). However, strict immobilisation
77 on a support matrix surface can be problematic, with difficulty attaching the enzyme to the
78 support structure and reduced activity due to distortion of the enzyme because of
79 interactions with the support (Rodrigues, Ortiz et al. 2013). Enzymatic membrane reactors
80 (EMRs) are an emerging technology and can serve as a form of enzyme immobilisation and
81 can involve binding of enzymes to larger support structures such as gel particles (Cabrera-
82 Padilla, Pinto et al. 2009) or the use of free enzymes maintained upstream of the membrane
83 (Cheison, Wang et al. 2006). EMRs work by retaining the enzyme and feed substrate on the
84 retentate side while allowing the newly formed PWSBs to permeate through the membrane,
85 generating a product stream with increased bioactivity. In this study, the enzyme is
86 introduced into the reaction vessel and retained upstream of the membrane on the
87 retentate side of the membrane, while being free and not bound to the membrane. EMRs
88 have been previously studied for the hydrolysis of various proteins with investigations of
89 fouling, flux, enzyme kinetics, amino acid sequence of peptides generated and process
90 modelling (Cheison, Wang et al. 2006, Cabrera-Padilla, Pinto et al. 2009, Eisele, Stressler
91 et al. 2013). However there are limited studies that examine the production of specific
92 bioactive peptides in an EMR while also monitoring bioactivity *in vitro*. An EMR-type setup
93 has been used specifically to produce peptides with DPPH activity using egg white as a
94 substrate (Jakovetić, Luković et al. 2015) However there have been no studies on whey-
95 derived DPP-IV bioactive peptides produced continuously in an EMR at the time of writing.
96 A study carried out (Cabrera-Padilla, Pinto et al. 2009) focused on generating ACE-inhibitory
97 and antioxidant peptides while using casein as a substrate doing so in an EMR with some
98 success. It was shown that the same substrate and enzyme, used under the same conditions,
99 in a batch system led to feedback inhibition of the *Bacillus lentus* alkaline peptidase by its
100 hydrolysed peptides, whereas using an EMR avoided this and increased productivity by 28%.

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

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

117

118 **2. Materials and Methods**

119 ***2.1 Materials***

120 Whey protein isolate (WPI) was supplied by Carbery Group, Cork. Corolase 2TS was donated
121 by AB Enzymes GmbH, Germany. Protamex was purchased from Novozymes, Denmark.
122 Polyethersulfone (PES) membrane sheets with a molecular weight cut off (MWCO) of 3kDa
123 were supplied by Synder Filtration, USA in 1m² sheets and were stored at 4°C until required,
124 and were then cut to size (0.0092m²) using a stanley blade. DPP-IV enzyme was supplied by
125 MerckMillipore, Ireland. NaOH, HCl, EtOH, MeOH, Tris, p-Nitroanalide, Gly-Pro-P-Analide,
126 DPPH, Trolox, Selenium Kjeldahl digestion tablets, H₂SO₄, KOH, Boric Acid were all supplied
127 by Sigma Aldrich, Ireland.

128

129 ***2.2 Choice of Processing Conditions***

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

133 enzyme manufacturers recommendations were considered for temperature, pH and dosing.
134 Membrane suppliers guidelines were also considered, in the case of processing temperature.
135 Temperature was chosen (55°C) to maximise flux while remaining within recommended
136 processing temperatures by both enzyme manufacturers. pH was chosen (7.5) based on flux
137 experiments and enzyme manufacturers recommendations. Enzyme dosing was based on
138 upper limit recommendations by enzyme manufacturers to avoid enzyme dosing being a
139 limiting factor in hydrolytic activity. They were 25mg Protamex/kg of substrate and 50ml
140 Corolase/kg substrate. The same processing conditions were used for all runs.

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

149

150 **2.3 Cross-Flow UF System and Procedure**

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

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

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

172

173 **2.4 Batch Runs**

174 For the batch experiments, 5L vessels were used with temperature control at 55°C. The
175 vessels were stirred at 300rpm with an overhead paddle stirring system. There was a limited
176 amount of foaming observed. 3.5L of WPI solution at a concentration of 5% w/w (protein
177 basis) was made up. Once the water bath and WPI solution were at a steady temperature of
178 55°C, the pH was adjusted to 7.5 using 2M NaOH and the enzyme was added at the
179 appropriate enzyme to substrate ratio (1:100 v:w for Corolase and 1:100v/v Protamex,
180 protein basis). The timer was started as soon as the enzyme was added and the pH was
181 maintained at 7.5 by manually adding 2M NaOH. The mass of NaOH added was recorded at
182 2 minute intervals and samples of the hydrolysate were taken every six minutes for the first
183 30 minutes, every 10 minutes for the following 30 minutes and every half hour for the next
184 three hours, with a total experimental time of four hours. The enzyme was deactivated by
185 heating the samples to 80°C for five minutes in a water bath. The samples were stored at -
186 20°C and utilised within 72 hours for all necessary testing. Permeate samples were also
187 tested for enzyme leakage.

188

189 **2.5 EMR Runs**

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

200 reaction vessel. After the 40 minute incubation period, the permeate flux was measured
201 every 60 minutes until the end of the experiment. Since a relatively low NMWCO was chosen,
202 3kDa membrane, it was important to ensure that the DH of the whey substrate was high
203 enough to allow a high mass flux of peptides. The level of bioactivity at that time was also
204 considered and was deemed to be approximately 90% of maximum bioactivity achieved in
205 the DPP-IV batch bioassay. For these reasons an initial incubation period of 40 minutes was
206 chosen for both enzymes in the EMR runs.

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

213

214 **2.6 Mass Balance, Mass Flux and Protein Determination**

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

224

225 **2.7 DPPH Assay**

226 2,2-diphenyl-1-picrylhydrazyl (DPPH) was dissolved in HPLC grade ethanol / MilliQ H₂O
227 (70:30) and diluted to a concentration of 120µmol. Care was taken to ensure the DPPH
228 solution was not exposed to light. Samples were prepared at a final concentration of 1mg/ml
229 (protein basis). and were centrifuged at 5000g for 10 minutes prior to assay. Samples
230 (0.5mL) were added to 1mL aliquots of the 120µmolDPPH solution, incubated in the dark at
231 room temperature for 45 minutes and their absorbance at 517nm measured. All samples
232 were analysed in triplicate and all absorbance readings were performed in triplicate

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

$$235 \quad RSA\% = [(TB - TS)/TB] \times 100 \quad \text{Eqtn. 1}$$

236 Where *TB* = test blank, *TS* = test sample

237

238 **2.8 DPP-IV Inhibition Assay**

239 The DPP-IV assay was carried out in triplicate as per Van Amerongen et al, (Aart, Catharina
240 et al. 2009) with some variations. Samples were diluted to final concentrations of 0.5, 1,
241 1.5 and 2mg/ml using 100mM Tris-HCl buffer, pH 8. Aliquots (25µl) of sample were pipetted
242 into a 96 well-plate and pre-heated to 37°C along with 25µl of Gly-Pro-P-nitroanilide
243 substrate. Once pre-heated, 50µl of the DPP-IV enzyme, pre-diluted to the appropriate
244 concentration based on its activity, was added to the well plate and the release of pNA
245 determined in a Thermo Scientific Varioskan LUX Multimode Microplate Reader at 405nm .
246 The activity of the DPP-IV enzyme preparation was confirmed prior to using it in the bioassay
247 by assessing the amount of P-Nitroanilide released using Gly-Pro_P-Nitroanilide as a
248 substrate. The enzyme activity deviated by less than 5% from the activity specified by the
249 supplier. Absorbance readings were taken after two hours of incubation. Test blanks,
250 positive control and negative controls were also analysed. The % inhibition was calculated
251 as per Equation 2.

$$252 \quad (1 - [(TS - TB)/(PC - NC)]) \times 100 \quad \text{Eqtn. 2}$$

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

255 IC₅₀ values were defined as the concentration of any product stream at which an inhibition of DPP-IV
256 enzyme is observed during the DPP-IV inhibition assay and is grams of protein per ml of solvent
257 (water). IC₅₀ values not measured directly. Further details below in section 2.10.

258

259 **2.9 Degree of Hydrolysis**

260 Degree of hydrolysis was measured using the pH-stat methods according to (Adler-Nissen
261 1986) with some modifications. Briefly, 2M NaOH was used to maintain the protein solution
262 and enzyme mix at pH 7.5 and the mass of NaOH solution added was recorded. Equation 3
263 below was used in the calculation.

264 $DH = B \times NB \times 1/\alpha \times 1/M_p \times 1/H_{tot} \times 100$ Eqtn. 3

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

267

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

272

273 **2.10 Comparison of Runs**

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

280

281 **2.11 Statistics and Replication**

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

290

291 **3. Results and Discussion**

292 **3.1 Batch Experiments**

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

295 The enzyme reaction begins quickly with a steep increase in DH within the first 6 minutes
296 and then slowing down somewhat while maintaining a steady rate of hydrolysis for the
297 remainder of both reactions. Corolase had the faster enzyme kinetics of tge two as is evident
298 from the higher DH, notable from minute 3. The general trends in RSA% results are similar
299 for both Protamex and Corolase, showing a significant decrease (P-value = 0.012 and 0.026,
300 respectively) in radical scavenging activity (RSA%) almost immediately upon commencement
301 of hydrolysis (compared with non enzyme-treated substrate) with no significant further
302 change thereafter ($\sigma = 1.47$ and 1.77 respectively). There was also no significant difference
303 between the two enzymes with respect to DPPH activity (P-value = 0.58). This reduction of
304 RSA% is somewhat unexpected as whey has been used as a feedstock for production of
305 antioxidative bioactive peptides in the past (Hernández-Ledesma, Recio et al. 2008, Peng,
306 Xiong et al. 2009, Nongonierma and FitzGerald 2013, Brandelli, Daroit et al. 2015). A
307 reduction in the activity upon hydrolysis suggests that there is no benefit to hydrolysis and
308 that, if anything, it is detrimental to the antioxidant potential of WPI. The non-hydrolysed
309 protein shows substantial RSA%, which has also been reported previously (Tong, Sasaki et
310 al. 2000, Peng, Xiong et al. 2009). Although Protamex and Corolase 2TS show a reduction in
311 RSA% upon hydrolysis, this does not necessarily mean that hydrolysis of whey protein will
312 always lead to the same reduction and it has been shown elsewhere that RSA% increases
313 with DH (Peng, Xiong et al. 2009, Peng, Kong et al. 2010, Kamau and Lu 2011). Despite no
314 change in bioactivity with increasing DH, samples generated in the EMR were still analysed
315 for RSA% for comparison to the batch hydrolysate. Again similar results were found in the
316 continuous process using the EMR. Therefore the IC₅₀ values with respect to DPPH were not
317 determined.

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

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

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

327 **3.2 EMR Experiments**

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

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

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

346 **3.3 Production Mass, IC_{50} and Cumulative DPP-IV Enzyme Inhibition**

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

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

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

365

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

377

378 **4 Conclusions**

379 Corolase 2TS and Protamex were both shown to be effective enzymes in producing peptides
380 with DPP-IV inhibitory affect using WPI as a substrate. Furthermore, a continuous method
381 of production using an EMR proved to be a more efficient way of producing DPP-IV inhibitory
382 peptides compared to standard batch processing methods. An increased production output
383 of 7.2% and 8.7% was achieved in continuous processing versus batch for Protamex and
384 Corolase respectively. Fluxes, specifically mass flux of protein fluctuated slightly for all
385 continuous runs, which can be overcome by engineering a more accurate feed dosing system
386 to ensure the total solids fed in (feed) versus out (permeate) are well balanced. Despite
387 some small fluctuations, generally flux trends remained stable and relatively high, with the
388 Protamex runs showing a slight but persistent reduction in flux over the entirety of the runs.
389 When comparing average fluxes to PWFs, fluxes averaged between 25% - 45% of the PWFs
390 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.

400

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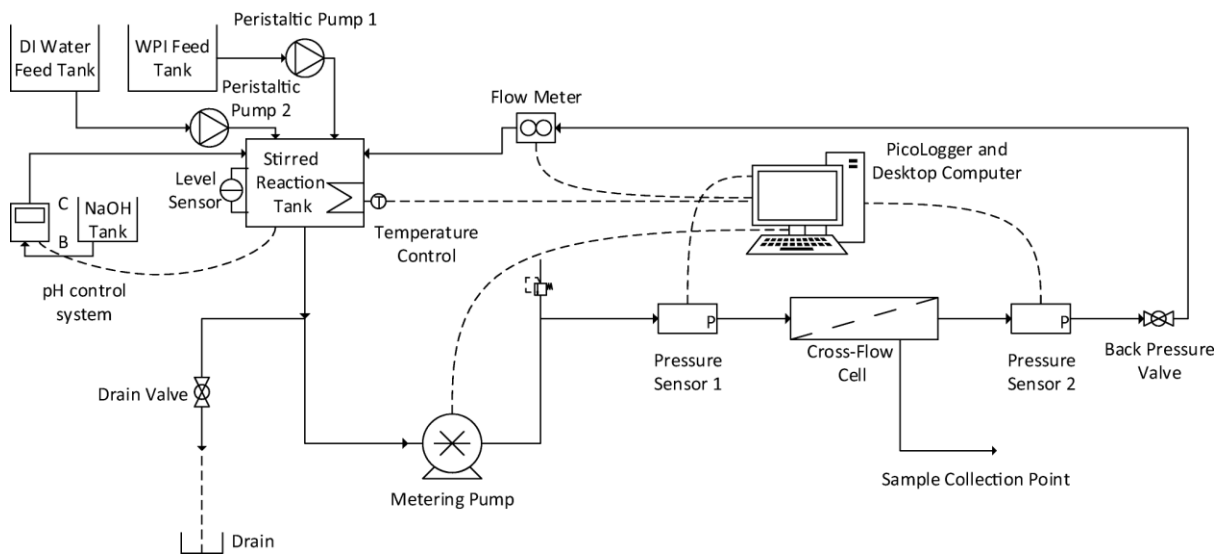
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515 **Figures**

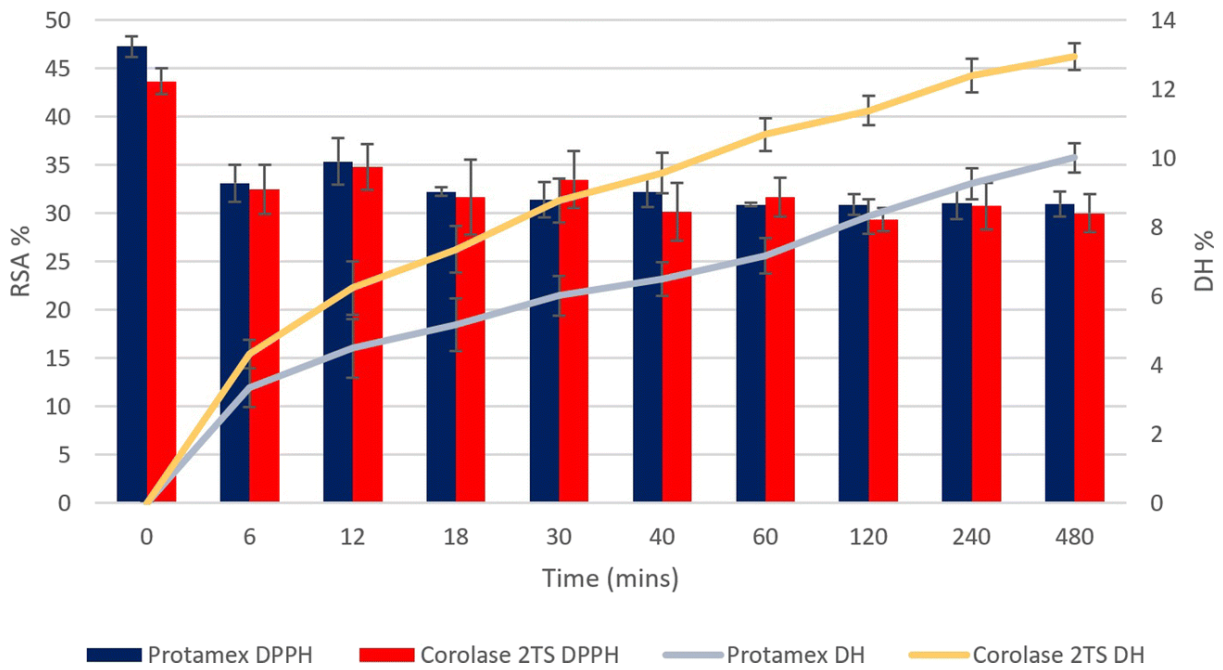


516

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

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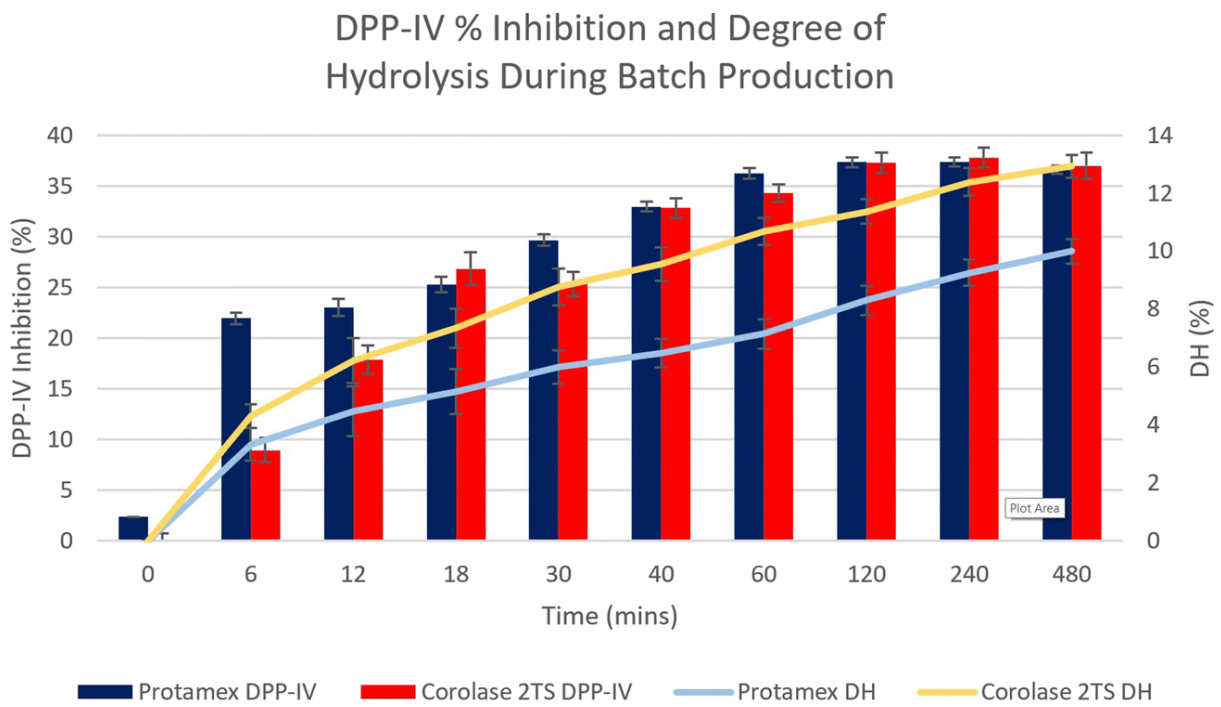
Radical Scavenging Activity and Degree of Hydrolysis During Batch Production



524
525

526 **Figure 2:** Evolution of Radical scavenging activity (RSA,%-bars) and degree of hydrolysis (DH,%-lines)
 527 during batch hydrolysis of WPI (5% protein basis) using Corolase 2TS, E:S = 1:100 (v/w protein basis) in
 528 red and Protamex, E:S 1:100 (w/w protein basis) in blue. Both batch experiments were carried out at
 529 55°C while maintaining pH at 7.5 using 2M NaOH. Sample concentration in DPPH assay 1mg/ml.
 530

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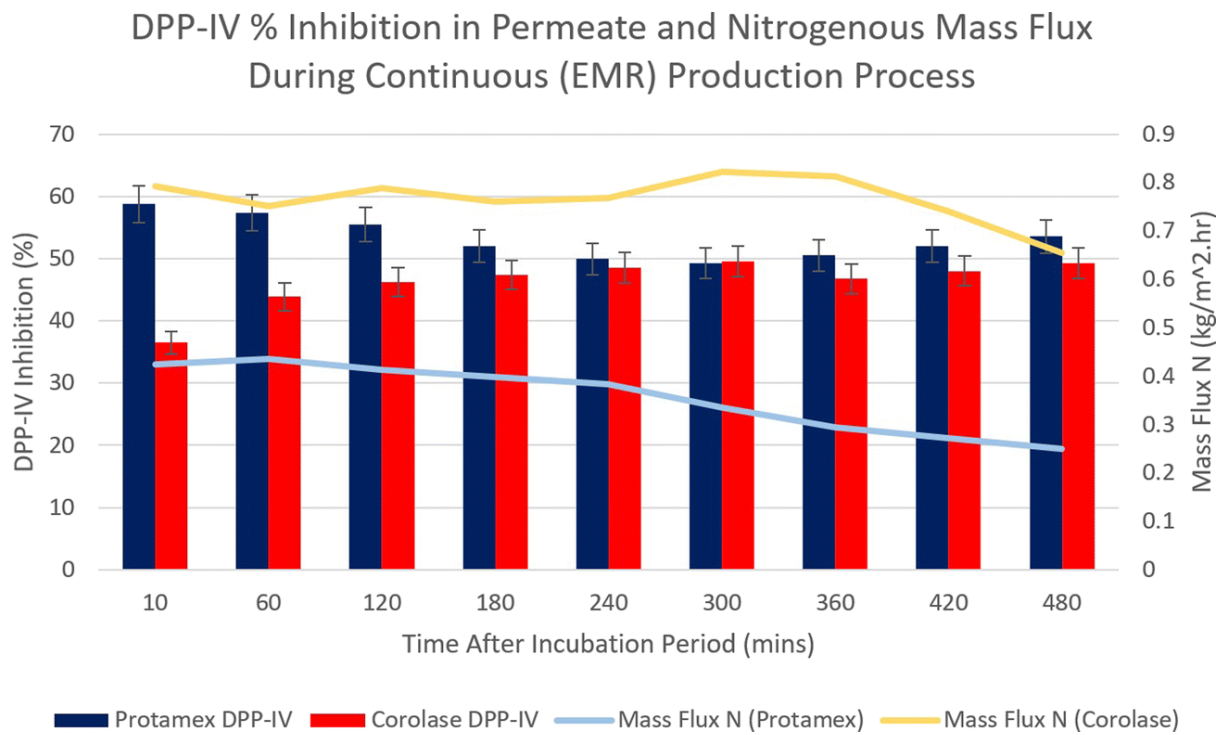
532

■ Protamex DPP-IV ■ Corolase 2TS DPP-IV — Protamex DH — Corolase 2TS DH

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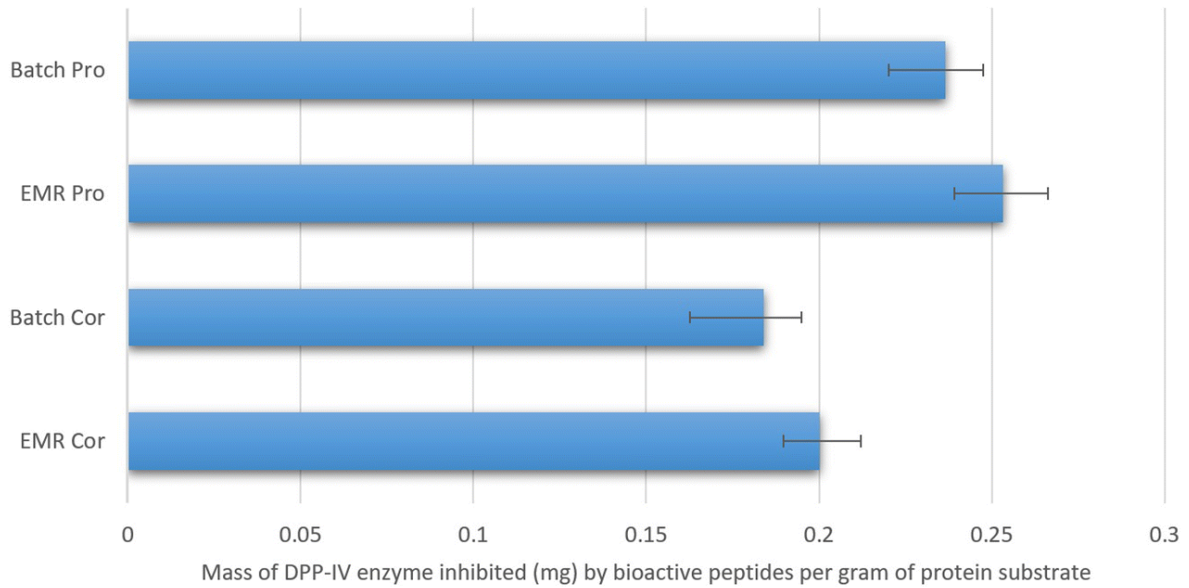
Figure 3: Evolution of DPP-IV inhibitory activity and DH during batch hydrolysis of 5% WPI (protein basis) using Corolase 2TS, E:S = 1:100 (v/w protein basis) in red and Protamex, E:S 1:100 (w/w protein basis) in blue. Both batch experiments were carried out at 55°C while maintaining pH-stat at 7.5 using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

537



539 **Figure 4:** Changes in DPP-IV inhibitory activity of permeate and mass flux during EMR Runs 1 and 2 using
 540 Corolase 2TS (blue) and Protamex (red) respectively. Conditions were the same as the previous batch
 541 experiments. Hydrolysis was allowed to proceed for 40 minutes (incubation period) before the pump
 542 was switched on and CFUF separation began using a 3kDa PES membrane. All data generated after
 543 incubation period.
 544

Overall comparison between all 4 runs for DPP-IV BAP production



545

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.

549

550

551